EXTENDING THE NUTRITIONAL GEOMETRIC FRAMEWORK TO THREE DIMENSIONS

I certify that all materials in this thesis that are not my own work have been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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ABSTRACT

Nutrition has pervasive effects on an organism's fitness, influencing traits across life history stages from early development and reproduction to ageing and lifespan. Consequently an understanding of these effects, and the mechanisms underpinning them, is of high importance in understanding variation in fitness. The current framework for studying nutrition (Nutritional Geometric Framework NGF) is limited in its usefulness in emerging questions in nutrition, and expanding the taxonomic reach of studies, because nutrients can only be studied in two dimensions (i.e. two nutrients can be studied simultaneously). In this thesis, I present an extension to the NGF with increased dimensionality, allowing more macronutrients to be manipulated simultaneously. To achieve this I apply principles of multivariate selection analysis to macronutrient consumption, allowing accurate analysis of the linear and non-linear effects of individual macronutrients, alongside correlational effects caused by interactions between macronutrients.

I then test this framework in a study of reproduction (Chapter 1) and lipid deposition and obesity (Chapter 2) in the cockroach *Nauphoeta cinera*, a species where nutritional effects have been examined previously, to test the practicality of the new framework, and assess new insight gained by this extended framework. In Chapter 1 I found all three macronutrients (protein (P), carbohydrates (C) and lipids (L)) to have substantial effects on fitness-related traits (pheromone expression and attractiveness in males and offspring number and gestation time in females), with sex-specific effects in trait maximisation, and new insight into traits compared to previous work using only two macronutrients (P and C). I also showed that males and females regulate their balanced macronutrient intake to almost exactly the same point when given a choice between diets.

In Chapter 2 I examined the effect of these macronutrients on lipid deposition in male and female cockroaches. Again I found large effects of all three macronutrients on lipid deposition, with sex-specific effects of different macronutrients maximising lipid deposition in males and females, despite the fact that both sexes regulate their intake to almost the same point. Across both experiments the shared regulated intake point was a closer match to female traits than male traits, suggesting the possibility for intralocus sexual conflict over the optimal intake of macronutrients.

Collectively, my thesis illustrates the effectiveness of the new multidimensional framework, and demonstrates that extra insight can be gained in previously studied areas that only examine the effects of two nutrients. Furthermore, it opens the door for wider application of the nutritional geometric methodology to longstanding questions in ageing and obesity, providing a more relevant framework for nutritionally complex species.

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AUTHOR'S DECLERATION

The research contributing to this thesis was conducted by Laurie Belcher (LB). All of the chapters presented in this thesis were written by LB with comments and editing from John Hunt (JH).

DEFINITIONS

Canonical analysis	The process of diagonalizing a matrix of non-linear coefficients of selection to produce eigenvectors.
Dietary restriction	A diet where some nutritional aspect, such as calories or protein, has been reduced compared to a baseline of usual diet.
Dietary choice	The macronutrient balance which individuals choose when allowed to mix between diets to regulate their macronutrient intake.
Eigenvector	Linear combinations of the separate traits where the greatest non-linear selection is occurring. Provides a perspective which reveals the major axes on which non-linear selection is occurring, aiding visualization of effects. The significance of eigenvectors can be quantified, as well as the strength and form of both linear (Θ_i) and non-linear (λ_i) selection acting on them.
Macronutrient regulation	The process by which individuals modulate their intake of macronutrients by consuming differing amounts of different foods which vary in macro nutritional composition.
Mixture triangle	A methodology allowing three macronutrients to be manipulate simultaneously by working with relative intake of macronutrients rather than absolute intake.
Multivariate selection analysis	A methodology using multiple regression models to quantify the linear and non-linear selection acting on traits. The accuracy of non-linear measures can be improved using canonical analysis.
Nutritional Geometric Framework	A state space modelling approach for manipulating two macronutrients simultaneously and examining the effects on measured traits.
Nutritional landscape	A graphic representation of the link between macronutrient intake and measured traits, created by mapping phenotypic data onto the nutritional space occupied by individuals
Nutritional rail	A straight line originating at the point (0,0) which represents a single ratio of one macronutrient to another and on which diets are chosen for experiments.

Nutrient signalling pathway	Pathways such as TOR and IGF which are sensitive to nutrient intake and can have large downstream effects through altering factors such as gene expression, metabolism, stress response, growth and autophagy.
Nutritional space	The area representing the full variation in macronutrient intake (on two or three axis) that can be obtained from diets available.
Post-ingestive regulation	Regulation of intake after food has been consumed. Can take the form of physical changes in gut morphology, changes in absorption efficiency of nutrients, and use of nutrients after absorption.
Protein leverage	A hypothesis that protein is the macronutrient where regulation is prioritized. This makes protein content of diets influential in determining intake of other macronutrients, with implications for traits such as obesity.
Regulated Intake Point	The place in the nutritional space which individuals will occupy when given a choice to modulate macronutrient intake by blending multiple diets that vary in macronutrient content.
2MT	2-Methylthiazolidine, a male sex pheromone released from the sternal glands of cockroaches.
3H2B	3-Hydroxy-2-Butanone, a male sex pheromone released from the sternal glands of cockroaches.
4E2M	4-ethyl-2-methoxyphenol, a male sex pheromone released from the sternal glands of cockroaches.

1. GENERAL INTRODUCTION

1.1 IMPORTANCE OF NUTRITION

Nutrition plays a central role in biology, shaping the distribution of individuals in space and time (Raubenheimer 2010), the expression of key life-history traits (Veiga et al. 1996; Hunt et al. 2004; Lee et al. 2008; Maklakov et al. 2008) and playing a part in traits as diverse as parental care (Hopwood et al 2013), thermoregulation (Coggan et al. 2011) and brain development (Nowicki et al. 2002). A substantial part of any individual's time budget is spent acquiring nutrients to meet these needs, which are often conflicting and form an integral part of evolutionary fitness (Lee et al. 2008). The traditional approach to nutrition has been a viewpoint of nutrition as a single currency (energy or calorie intake) (Stephens & Krebs 1986). This view has, however, recently been challenged in many key areas of research (Simpson & Raubenheimer 2012). It is clear now that nutrition cannot be accurately modelled as a single currency as its constituent parts, macronutrients, micronutrients, vitamins and minerals all have widely varied functions and important consequences for fitness (Simpson & Raubenheimer 2012). For example, prey species can vary greatly in macronutrient composition (Fagan et al. 2002; Jensen et al. 2011; Raubenheimer 2011) and predators are able to balance their intake of these macronutrients (and not just calories) to optimize fitness (Mayntz et al. 2005; Jensen et al. 2012; Hewson-Hughes et al. 2013). Furthermore, the balance of macronutrients, particularly protein and carbohydrates, provides a much better explanation of lifespan extension than caloric restriction in many species (Lee et al. 2008; Maklakov et al. 2008; Fanson et al. 2009; Jensen *et al*. 2015). Rather than facing the simple challenge of obtaining enough energy, individuals face complex nutritional challenges, balancing intake of diets of varied macronutrient content to obtain an optimal intake. Given the importance of nutrition to evolutionary fitness, this added complexity deserves greater theoretical and empirical attention (Simpson & Raubenheimer 2012).

1.2 THE NUTRITIONAL GEOMETRIC FRAMEWORK

The current "gold standard" approach for investigating the effects of nutrition is the nutritional geometric framework (NGF) (Simpson & Raubenheimer 1993). The NGF is a state-space modelling approach which works on two main principles: (*i*)

nutritional intake takes place along multiple dimensions, represented by specific macronutrients (i.e. protein, carbohydrates and lipids) and (*ii*) to place nutrition in the context of evolutionary fitness, the consequences of intake need to be mapped onto the the intake of nutrients. The NGF can therefore work with simultaneous manipulations of multiple macronutrients, and examine the consequences of intake on any number of measureable fitness traits. The independent effects of individual macronutrients can be quantified, as well as any interactions between macronutrients. A typical study using the NGF uses 24 diets, chosen to represent a broad spread of the available nutritional landscape of two macronutrients, usually protein and carbohydrate (Figure 1.1). These diets sit on nutritional rails of fixed macronutrient ratio (in the example, there are six different macronutrient ratios). Furthermore, along these nutritional rails, diets are arranged so that they vary in total nutrition (in the example, there are 4 diets per nutritional rail that have 12%, 36%, 60% and 84% total nutrition). These diets on different nutritional rails can therefore be connected by isocaloric lines (lines connecting diets with equal calories, Figure 1.1). Collectively, this design creates a geometric array of diets in maconutrient space (Figure 1.1). Each diet can then be fed individually to a series of animals and any number of fitness traits (i.e. lifespan, reproduction) measured. Response surface analysis (Lande & Arnold 1983) can then be used to examine the linear and nonlinear (i.e. quadratic and correlational) effects of nutrient intake on the fitness traits that have been measured. Importantly, the geometric design of diets enables the independent and combined effects of specific nutrient intake on fitness traits to be statistically partitioned from the overall effects of total nutrition (or calories). Measures of fitness traits can also be mapped onto nutrient intake data using thin-plate splines to construct a nutritional landscape (Figure 1.2) to visualize the location of fitness optima. This data is then often linked to nutritional choice trials, where individuals are given the choice between various diets in the nutritional space, which they can feed freely from to balance their intake of nutrienst towards a regulated intake point. This can then be mapped onto the nutritional landscape to determine if animals are able to regulate their intake of nutrients optimally for the expression of fitness traits (Lee et al. 2008; Maklakov et al. 2008; South et al. 2011).

The NGF has been integral in furthering understanding of the role of nutrition in numerous areas of evolutionary research. It is well established that dietary restriction

(DR) extends lifespan in a diverse array of species (Nagakawa et al. 2012), although there is a lack of consistency, with some studies finding no effect of DR (Kirk 2001; Cooper et al. 2004). The problem is partly due to subtle variations in experimental diets (Piper et al. 2014), which combined with a lack of standard experimental design may have led to discrepancies in effect size (Nagakawa et al. 2012). The NGF has provided a standard framework of diets and methodology that has already provided great insight into the role of DR in ageing. For decades the prevailing view was that a restriction of calories caused increasing lifespan (McDonald & Ramsey 2010), a theory that still persists in explaining effects of DR on mammalian lifespan (Masoro 2006, 2009). It was only by using the NGF to separate the effects of macronutrient intake and calorie intake that this theory could be rebuked, and the importance of protein brought forward as the prevailing view (e.g. Lee et al. 2008).

The NGF has also led to a challenge to the dogma of foraging theory and traditional views of nutrition, where classical models predict that individuals will maximise energy intake to order to optimize fitness (Emlen 1966, Stephens & Krebs 1986). Using the NGF to show predator macronutrient balancing (Mayntz et al. 2009; Jensen et al. 2012; Hewson-Hughes et al. 2012) has opened up new questions in the nutritional ecology of predators, and has implications for optimal foraging theory more generally.

Given the fundamental importance of nutrition, it is continually being applied to new areas and this has been aided by the NGF. Recent work has begun to investigate the role of macronutrient balance in immunity (Cotter et al. 2011; Ponton et al. 2013) and optimization of livestock feeds (Ruohonen et al. 2007; Zhang et al. 2012; Cowieson et al., 2014) as well as bringing the framework to studies in the field (Felton et al. 2009; Rothman et al. 2011; Johnson et al. 2013). A key strength of the framework is that it provides consistency across studies, which is much needed in many areas of research. An example of this is the evolution of obesity, where arguments continue on the relative importance of carbohydrate and lipids in promoting obesity. The NGF has provided important evidence that macronutrient balance in key to the evolution of obesity (Solon-Biet et al. 2014).

1.3 LIMITATIONS OF THE NGF

The NGF has undoubtedly revolutionised the way nutrition is viewed, with its main strengths being the way it incorporates the complexities of a multidimensional landscape varying in several macronutrients, allows fitness consequences to be mapped onto those landscapes, and provides consistency across different studies. There are, however, several key limitations that restrict the usefulness of the NGF as the field progresses to new fields and new species.

The first major limitation is one of interpolation. As explained above, the typical NGF study uses diets on six nutritional rails (Figure 1.2) of fixed macronutrient ratio (e.g. Lee et al. 2008; South et al. 2011). Fitness traits are then measured for individuals consuming these diets, but the fitness consequences of nutrient intake between the nutritional rails has to be estimated by interpolation. This is because nutritional intake data only falls along the nutritional rails: an individual can eat more or less diet which would push it up or down along the length of the nutritional rail, but it cannot eat towards different regions in nutritional space**.** This can make detecting subtle variation in peaks of fitness traits difficult or can even lead to the incorrect placement of peak(s), both of which can have important consequences when assessing the optimality of dietary regulation. Furthermore, incomplete coverage of diets in nutritional space means there are many areas of the nutritional space that are not represented by data where interesting effects could occur, such as with extreme diets at the edge of the space. Understanding the consequences of consuming these extreme diets can help make sense of macronutrient regulation, where some macronutrients may be more tightly regulated that others. This could be due to the severe consequences for traits, such as lifespan and obesity, where over and under consuming this macronutrient to extreme levels (Simpson & Raubenheimer 1999; Skorupa et al. 2008). A second limitation is that by design,the intake of nutrients across nutritional rails are strongly correlated. This presents problems with determining whether the effects of nutrient intake on fitness traits are direct or indirect (correlational). In short, response surface analysis (which is effectively a multiple regression based approach) is vulnerable to strong correlations between predictor variables (i.e. nutrient intake): a statistical issue referred to as multicollinearity. Multicollinearity has the potential to mask “true” patterns or effects in the data. This is particularly important when examining how dietary preferences may evolve, and what drivers are responsible (Reddiex et al. 2013). A final limitation

of the NGF as it is typically employed is dimensionality. Most studies currently use protein and carbohydrate, or sometimes protein and non-protein forms of energy (Gosby et al. 2011; Rothman et al. 2011). This has been considered sufficient for many taxa, particularly insects, where nutritional ecology is relatively simple (Piper et al. 2011). Problems, however, arise when investigating species with more complex nutritional environments, for example ones featuring all three macronutrients (protein, carbohydrate and lipids) or an array of important micronutrients. The NGF has shown great promise in providing answers to long standing questions related to human health and lifespan. Testing theories relies on first testing predictions in animal models such as mice and non-human primates, but for these studies to be nutritionally relevant a framework which can simultaneously manipulate multiple macronutrients is required. As well as boosting the number of macronutrients studied, increased dimensionality would improve the framework on a finer scale, as macronutrients are not the basal components of nutrition, being themselves made of constituent parts such as amino acids. Individual amino acids may play a role in effects of protein, such as dietary restriction and ageing (Grandison et al. 2009), but the sheer number of amino acids mean the current framework is unable to full investigate this, as studies would need to manipulate many amino acids simultaneously, as there are likely to be significant interactions (Grandison et al. 2009).

1.4 MIXTURE TRIANGLES

The need for increased dimensionality in the NGF has long been recognised (Raubenheimer & Simpson 1999) and work has already begun to widen the framework to more than two nutrients. Right angled mixture triangles (Raubenheimer, 2011) have already been used many times to study three macronutrients simultaneously, and in particular have been used to investigate optimal diets in aquaculture (Ruohonen et al. 2007; Zhang et al. 2012). This framework differs from the traditional NGF in that it focuses on relative amounts of macronutrients in the diet rather than focussing on the absolute amounts of macronutrients consumed. As such the available nutritional space forms a triangle with any point within the triangle having a sum of 100% nutrition (Figure 1.3). This is highly useful in optimizing animal feeds, and has helped alleviate one issue with the NGF in that it is of limited use in the field where absolute intake is difficult to

calculate and relative intake comparatively much simpler (Johnson et al. 2013). Mixture triangles have since been applied with much success in field studies, and have enabled nutritional choice and relative nutrient regulation to be estimated in these scenarios (Raubenheimer et al. 2015). However, this approach suffers many of the same limitations as the traditional NGF. Interactions involving total nutritional content, which is always held constant in mixture triangles, also cannot be examined. These can be crucial as an individual's total nutritional demands commonly vary with different physiological processes (i.e. mating) and developmental stages (i.e. sexual maturation) (Jensen et al. 2012; Kohler et al. 2012; Lee et al. 2013). Furthermore, over-and under ingestion of certain macronutrients can occur even at the same point in a mixture triangle depending on the total nutritional level. This could result in inconsistency of target intake points dependant on the total nutritional level, meaning that experiments need to repeat experiments at different nutritional levels to assess the accuracy of conclusions (e.g. Solon-Biet et al. 2014). Mixture triangles have provided great insight in new areas of nutrition, but as a general framework for advancing the integrative study of nutrition, they are still lacking in certain key areas.

1.5 AIMS AND OBJECTIVES OF RESEARCH

The principal aim of my research is to extend the NGF to three dimensions to allow protein, carbohydrate and lipids to be manipulated simultaneously and both the individual effects and interactions between macronutrients to be quantified along with the effects of total nutrition. Furthermore, I will present both the theoretical aspect of this framework, and empirical data applying the framework to several experiments.

The NGF currently uses response surface methodology, but there is an opportunity to extend this analytically to allow increased dimensionality, by using established principles from quantitative genetics and multivariate selection analysis. Researchers working on selection analysis have long recognised the need for multivariate approaches (Thompson, 1977: Lande & Arnold 1983). Selection acts on combinations of traits, as opposed to individual traits, due to the fact that traits are linked due to linkage disequilibrium and pleiotropy (Lande & Arnold 1983; Brodie et al. 1995; Brooks & Endler 2001). This is analogous to nutrition, where it is known that macronutrients don not have effects on their own, rather effects are due to the

balance of macronutrients and interactions between them. In selection analysis this issue has been resolved by separating direct and indirect selection acting on traits using second order multiple regression and canonical analysis (Phillips & Arnold 1989) and this analysis has been used to great effect in selection analysis (Chenoweth & Blows 2005; Ower et al. 2013; Steiger et al. 2013) and this approach can be easily transferred to study the effects of multiple macronutrients. This counters the large limitation of restricted dimensionality, but in order to counter the other limitations (interpolation, lack of coverage and multicollinearity) further changes need to be made. This can be done by producing substantially more diets than in a tradition NGF study, and spreading them randomly across the full nutritional space. Fewer replicates of each diet are required, as this design is instead centred on producing a full spread of the nutritional space.

Chapter 1 presents the theory of the framework extension, as well as results of a study applying the framework to a study of the effects of macronutrient intake on male and female reproduction in the cockroach *Nauphoeta cinerea*. Chapter 2 presents the results of a study applying the framework to a study on male, female and offspring fat deposition in the same species, as well as nutritional choice and regulation at the level of three macronutrients.

The cockroach, *Nauphoeta cinera*, has been used previously to investigate the effects of macronutrientsusing the NGF, with studies finding that males prefer a high carbohydrate diet which increases their attractiveness to females due to maximal expression of sex pheromones (South et al. 2011). Further work has examined the role of protein and carbohydrate intake on sperm production and viability and male fertility(Bunning et al. 2015). This study concluded that males are choosing a diet that appears to be intermediate between that which optimizes pre-copulatory mating success through attractiveness to mates on a high carbohydrate biased diet and post-copulatory success through sperm number and fertility on a more protein biased diet. Both of these studies used the two macronutrient (protein and carbohydrates) NGF approach, and part of the aim of this thesis is to assess the extra insight that can be gained using a three macronutrient approach that includes lipids. Carbohydrates are stored as lipids in cockroaches (Nation, 2001) and are important precursors for sex pheromones (Chase et al. 1992) and so may simply be exchangeable with carbohydrates in diets, or may have completely different

functions and effects due to potential difference in costs of under and over consuming these nutrients (Warbrick-Smith et al. 2006), energy density (Rolls, 2000), and physiological impacts (Arrese & Soulages 2010).

1.6 POTENTIAL APPLICATIONS

This extended framework has large potential both in areas in which nutritional geometry has already been applied, and in emerging areas where it has yet to be implemented. There is increasing interest in the potential for dietary restriction (DR) to extend healthy lifespan in humans, in light of the effects observed across a broad range of taxa already tested (Nagakawa et al. 2012). Current manipulations are unable to represent the full nutritional space of protein, carbohydrates and lipids (Weindruch et al. 1986, Masoro et al. 1989) and recent work using mixture triangles has hinted that effects may be conserved in mice (Solon-Biet et al. 2014).

Protein seems to be the macronutrient most important in explaining the effects of DR (Lee *et al*. 2008; Simpson & Raubenheimer 2009; Sun et al. 2012) but there is also evidence that specific amino acids, particularly methionine, play a key role (Orentreich et al. 1993; Miller et al. 2005, Grandison et al. 2009). The new framework will provide the ability to simultaneously manipulate many amino acids, improving on the individual manipulations of previous studies. By answering emerging questions about the universality of DR, and the causal agent behind it, the new framework can provide great insight into this field.

The extended framework also has potential to provide clear and consistent answers to question in human health, particularly obesity. There is still much debate about the causal nutritional profile of obesity (Samaha et al. 2003; Austin et al. 2011; Te Morenga et al. 2013: MacGregor & Hashem 2014) with single macronutrient or energy associations still prevalent (Swinburn et al. 2011). Recent evidence, using mixture triangles, has suggested however that macronutrient balance is again the most important factor in fat deposition of mice, rather than any individual macronutrient (Solon-Biet et al. 2014). With the sum of evidence increasingly pointing towards diet as a more important driver of obesity than exercise levels (Westerterp & Speakman 2008; Swinburn et al. 2009a) it is ever more important to

understand the phenomenon of obesity, and the extended framework provides the opportunity for a rigid and consistent methodology to assess these effects and importantly compare between studies. Focussing on balance of macronutrients and their interactions rather than levels of any one macronutrient can provide great insight, and the NGF in its current form has been unable to contribute significantly here in the past due to its restriction to two macronutrients.

The ability to study a wider range of taxa using three macronutrients simultaneously also is also likely to provide significant insight. The universality of regulatory rules and dietary consequences can be tested, and differences between species can be investigated by using the standardized procedure I outline in my thesis. Many studies on higher order taxa have combined carbohydrate and lipid into ‘non-protein energy’ however differences in both energy density and function (Arrese & Soulages 2010) of carbohydrates and lipids mean important information is likely to be missed. This aspect is likely to be particularly relevant to carnivores and predatory insects, where nutritional ecology has been found to be substantially more complex than previously assumed (Raubenheimer, 2011; Jensen et al. 2012).

FIGURE LEGENDS

Figure 1.1. Protein and carbohydrate content of the 24 experimental diets traditionally used for studies in nutritional geometry. Diets are placed on one of 6 nutritional rails, of varied ratio of protein to carbohydrate (shown as solid lines). Dashed lines are isocaloric lines, with all diets on a line sharing the same total caloric content.

Figure 1.2. Figure taken from Bunning et al (2015). An example of a nutritional landscape used to visualise effects of diet on focal traits. Unfilled circles represent daily intake of protein and carbohydrate, which is constrained to a nutritional rail. Red areas correspond to high male fertility, and blue areas low male fertility

Figure 1.3. Figure adapted from Raubenheimer 2011. Representation of the mixture triangle methodology for manipulating three macronutrients simultaneously, showing composition of protein, carbohydrate and lipid. This approach uses relative proportions of macronutrients, such that any point in the available nutritional space (eg. filled black circles) have a total nutritional content of 100%

Figure 1.1

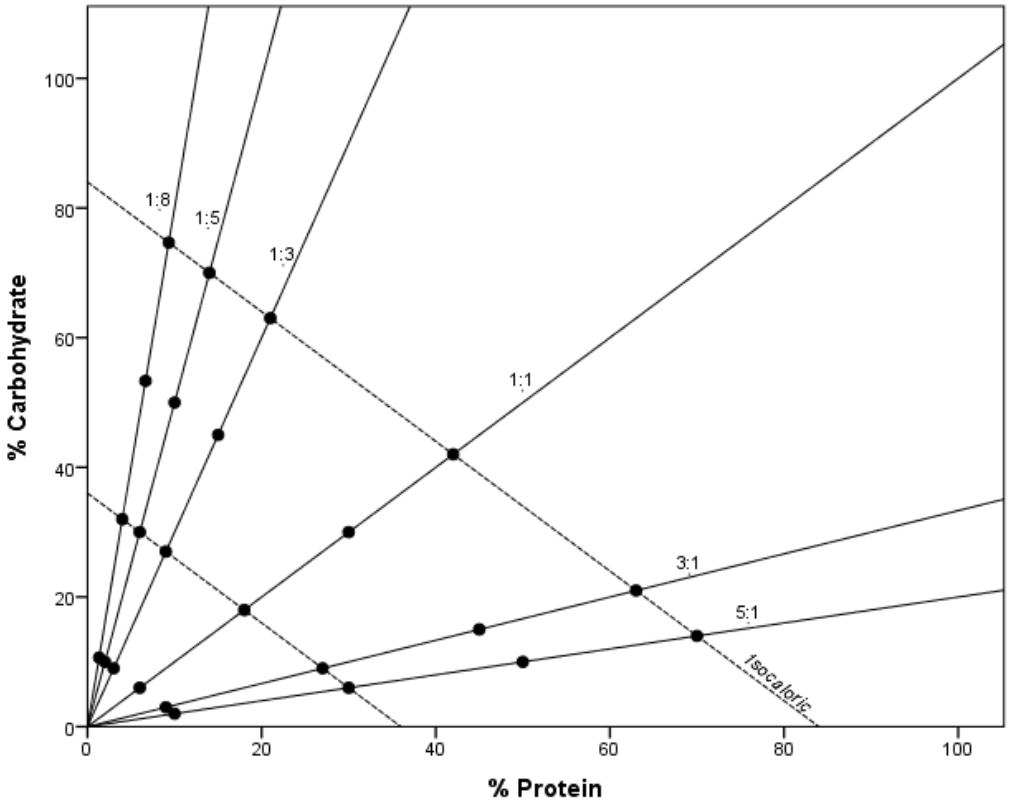

% Carbohydrate
% Protein
0
20
40
60
80
100
1:8
1:5
1:3
1:1
3:1
5:1
isocaloric

Figure 1.2

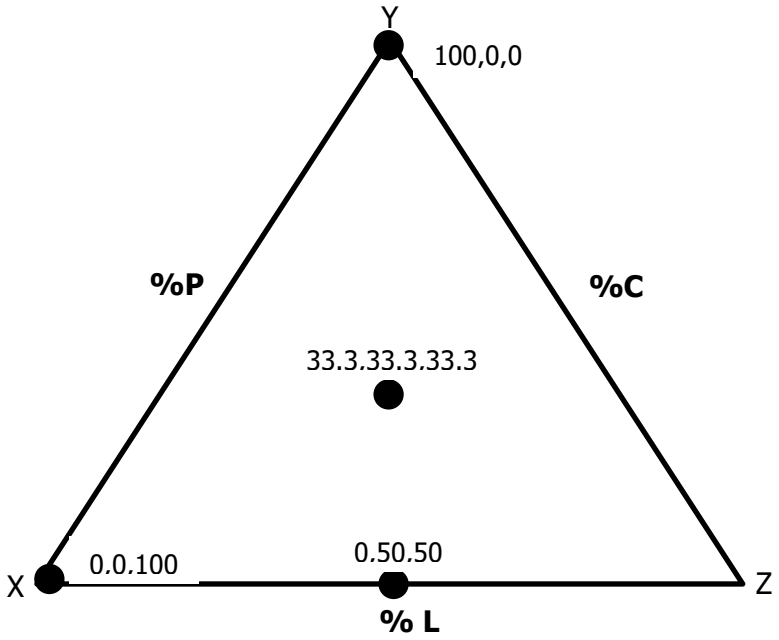
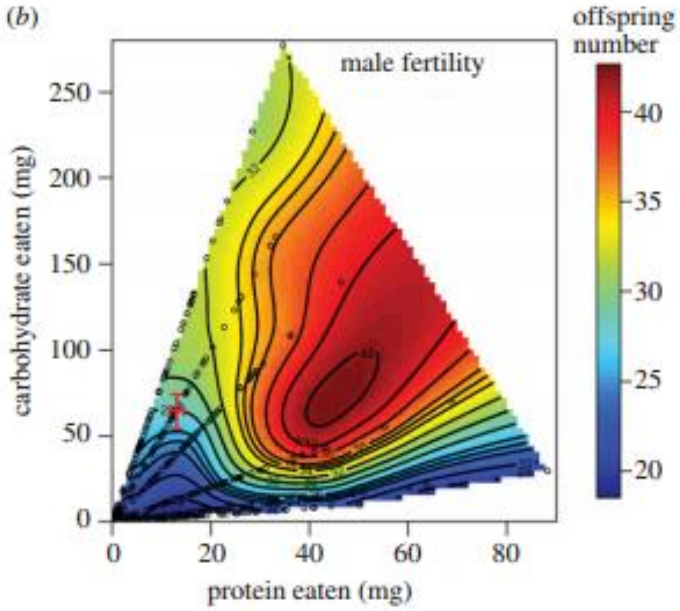


Figure 1.3


(b)
male fertility
offspring number
40
35
30
25
20
carbohydrate eaten (mg)
250
200
150
100
50
0
0
20
40
60
80
protein eaten (mg)

2. THE IMPACTS OF MACRONUTRIENT INTAKE ON REPRODUCTIVE FITNESS: A NEW THEORETICAL FRAMEWORK

2.1 ABSTRACT

Macronutrient intake plays an important role in a large range of fitness related traits, from fecundity to longevity. Many studies have used the Nutritional Geometric Framework (NGF) to examine these effects in two macronutrients, providing insight into the importance of a balanced macronutrient intake over calories, and the rules of nutrient regulation. This framework, however, suffers from several key limitations: weak interpolation of data across diets spread on a geometric array, correlation between dietary ratios, omission of extreme diets, and restricted nutritional relevance in many taxa. These limitations reduce the usefulness of the NGF in key and emerging areas of nutrition. Here I present an extension of the NGF with increased dimensionality using the principles of multivariate selection analysis, allowing the effects of three macronutrients (Protein (P) carbohydrates (C) and lipids (L)) to be manipulated and partitioned simultaneously. Furthermore I use this new framework to provide further insight into studies of reproduction in the cockroach *Nauphoeta cinerea*. I found the intake of all three macronutrients to have large effects on fitness traits, with differences within and between sexes. Female offspring number was maximised at high C and P intake, and gestation was shortest with high intake of all three macronutrients. Male sex pheromones and attractiveness were both maximised at high intake of both carbohydrates and lipids, with male fertility being much more responsive to high protein intake. Males and females were found to share a remarkably similar macronutrient intake ratio under choice, with this ratio being much closer to the optima for female traits than male, raising the possibility of sexual conflict over nutrition or sex differences in regulatory ability. This framework has broad potential in a range of areas, such as understanding the role of

macronutrients in lifespan extension, investigating how and why individuals regulate their intake and improving the taxonomic spread of research.

Keywords: Nutritional Geometry, *Nauphoeta cinerea*, diet choice, multivariate selection analysis, reproductive fitness

2.2 INTRODUCTION

The amount of nutrients an individual consumes is known to have significant effects on the primary components of fitness; reproduction and survival (Lee et al. 2008; South et al. 2011; Simpson & Raubenheimer 2012). Diet is also an important factor influencing life history and sexual selection, with many traits being condition dependent (Rowe & Houle 1996; Cotton et al. 2004). Traditionally these effects have been examined by manipulating the energy content of food (Chapman & Partridge 1996; Rastogi et al. 2006), or single nutrients at a time (Davies et al. 1983; Iwasaki et al. 1988). This approach, however, fails to recognise that organisms need a variety of macronutrients for different functions, making decisions more complex than purely maximising energy intake.

The Nutritional Geometric Framework (NGF) provides a method for understanding the effects of specific macronutrients on key life history traits (Simpson & Raubenheimer 1993). The framework uses a multi-dimensional nutritional space varying in two macronutrients (most often protein and carbohydrate intake), with dietary treatments spread across this space in a geometric array (Figure 2.1). A series of animals are then restricted to single diets from this array in a “no-choice” experiment and the intake of nutrients and fitness-related traits measured in these individuals. Response surface methodologies (Lande & Arnold 1983) can then be used to quantify the linear and nonlinear effects of nutrient intake on the traits of interest and partition these effects from those due to the intake of calories. These effects can be visualized by mapping traits onto the nutrient intake data using thin-plate splines to construct a nutritional landscape. Further experiments presenting animals with a choice of diets to calculate target intakes are often used to assess the optimality of choice (Lee et al. 2008; Maklakov et al. 2008), and what traits and

nutrients are prioritized when resources are constrained (Raubenheimer & Simpson 2003; South et al. 2011).

By simultaneously studying the effects of two macronutrients, as well as the interactions between them, NGF has spearheaded advances in a diverse range of biological fields (Simpson & Raubenheimer 2012). For example, using a standard framework of diets and methodology the NGF has shown that a reduction in protein intake is more important than a reduced intake of calories in explaining the effects of dietary restriction on lifespan (Lee et al. 2008; Solon-Biet et al. 2014). Similarly the framework has been used to show that predators target a balance of nutrients (protein and lipids) when foraging, rather than just maximizing the intake of energy (Mayntz et al. 2009; Jensen et al. 2012; Hewson-Hughes et al. 2012). Due to the universal importance of nutrition there are countless areas where the NGF has provided insight into the role of specific macronutrients, such as immunity (Cotter et al. 2011; Ponton et al. 2013), obesity (Simpson & Raubenheimer 2005; Gosby et al. 2011) and development (Sentinella et al. 2013). The NGF standardises research with fully defined diets and is widely applicable through the mapping of a variety of fitness traits onto the nutritional landscape (Lee et al. 2008; Cotter et al. 2011). There are, however, several key limitations hindering the ability of this approach to answer more complex, emerging questions.

The first limitation of the NGF is poor data coverage in nutritional space. A typical NGF study uses 24 diets that span 6 macronutrient ratios (most often protein and carbohydrates), known as nutritional rails (solid lines in Figure 2.1; Maklakov et al. 2008; South et al. 2011). Diets are spread evenly along each nutritional rail so that they differ as they move away from the origin they increase in caloric content (Figure 2.1). On different nutritional rails, however, diets are matched for caloric content (dashed “isocaloric” lines in Figure 2.1), enabling the effects of nutrient and caloric intake to be statistically separated. Fitness-related traits are mapped onto this nutrient intake data, but as individuals are restricted to feeding on a single diet, nutrient intake data is necessarily constrained along the nutritional rails. Consequently, large regions of the nutritional landscape that exist between these nutritional rails are not populated by data meaning that the relationship between nutrient intake and traits must be interpolated. This could lead to inaccuracies in the location and size of any nutritional optima (Maklakov et al. 2008), as well as missing

subtle variations in the nutritional landscape, such as the degree of curvature of the peak. A second, related limitation of designing artificial diets that a positioned along fixed nutritional rails is that there will inevitability be a strong positive correlation between the intakes of nutrients (Figure 2.1). Strong correlations between predictor variables, known as multicollinearity, is well known to hinder the ability of response surface methodologies (especially multiple regression analysis) to partition the independent effects of the predictor variables on the response variable (Mitchell-Olds & Shaw 1987). In theory, this could result in the misinterpretation of the relative importance of specific nutrients on the traits being examined. A third limitation of the NGF is the omission of extreme diets when using structured diets that are presented in a geometric array (Figure 2.1). The frequency of data points at the extreme of the distribution for predictor variables is known to have important consequences for estimating the direction and curvature of a responses surface (Mitchell-Olds & Shaw 1987). This is particularly important for nutritional data where the frequency of data point at the extreme of the distribution is likely to influence the location of the nutritional optima. A final limitation of NGF is the biological reality of using only two nutrients. While a two dimensional approach has been sufficient for species, such as *Drosophila*, which naturally obtain most of their nutrition from only protein and carbohydrates, most species also obtain much of their nutrition from lipids (Piper et al. 2011) and a variety of different micronutrients (e.g. salts, vitamins) are often required for normal health and reproduction (Trumper & Simpson 1993; Fanson & Taylor 2012; Mehrad et al. 2012). Thus, is likely that only focussing on two nutrients at a time will seriously underestimate the complexity of nutrition and clearly highlights the need for a multivariate approach.

Currently, mixture triangles are the only established methodology allowing three macronutrients to be manipulated simultaneously (Raubenheimer 2011), being used primarily to optimize commercial animal feeds (Ruohonen et al. 2007; Zhang et al. 2012; Hewson-Hughes et al. 2012, 2013). They are very useful in this context, where space and the replication of diets are restricted compared to model organisms in the laboratory. Mixture triangles are also useful in studying nutritional choices in the wild, as they use relative proportions of macronutrients rather than absolute amounts consumed, overcoming the difficulties of accurately measuring intake in the field (Johnson et al. 2013). There are, however, key

limitations to mixture triangles. The sum of the percentage composition of the macronutrients must always equal 100%, resulting in the ability to look at interactions between macronutrients, but not interactions with total nutrition level, which are important. Intake targets vary with development and the reproductive status of individuals (Jensen et al. 2012; Lee et al 2013) causing interactions between nutrients and total nutritional value. The costs of over and under ingestion could also result in tight regulation of specific nutrients (Jensen et al. 2013; Kohler et al. 2012), leading to different intake targets depending on the nutritional level of the triangle. This may result in the need to repeat experiments at different total nutritional levels to obtain accurate information about the independent effects of macronutrient intake and total nutrition, as well as their interaction (Solon-Biet et al. 2014). Most existing studies only examine the effects of macronutrients at two different levels of total nutrition (i.e. low and high), which provides very little scope to understand the complexity of any interactions (Solon-Biet et al. 2014; Le Couteur et al. 2014). Here, I use established principles from multivariate selection analysis to extend the NGF to three dimensions (i.e. three nutrients). It is well established that selection does not act on individual traits *per se*, but rather on combinations of correlated traits (Lande & Arnold 1983). Natural correlations between traits can make it difficult to separate the effects of selection directly targeting a given trait (i.e. direct selection) from selection operating on other correlated traits (i.e. indirect selection). Multivariate selection analysis deals with this issue analytically (Lande & Arnold 1983) but phenotypic manipulations that break the natural covariance between traits has also proved extremely useful in estimating the complexity of selection (e.g. Brooks et al. 2005; Bentsen et al. 2006). By viewing the intake of macronutrients in the same way as phenotypic traits, artificial diets can be created that vary in both macronutrient content and total nutrition and where the content of the different macronutrients are uncorrelated. Multivariate selection analysis can be used to estimate the linear and nonlinear effects of nutrient intake on a range of desired traits and canonical analysis, an approach commonly used to accompany multivariate selection (Phillips & Arnold 1989), can be used to locate and visualize the combination of nutrients (known as nutritional vectors) that maximise these traits.

To empirically test this technique I constructed 300 artificial, holidic diets differing in protein (P), carbohydrate (C) and lipid (L) content and examined the

effects of these nutrients on a range of fitness-related traits in male and female cockroaches (*Nauphoeta cinerea*) when restricted to a single diet (Experiment 1). Next, we presented cockroaches with the choice between three diets differing in P, C, and L content to determine how the sexes regulate their intake of macronutrients and whether this enables them to optimize trait expression (Experiment 2). Previous work on this species used the NGF to vary protein and carbohydrate intake and found that sex pheromone expression in males is regulated predominantly through the intake of carbohydrates and this increased their attractiveness to females (South et al. 2011). Importantly, when provided with dietary choice, males actively consume a high carbohydrate diet to maximise their attractiveness (South et al. 2011). This work, however, did not examine the intake of lipids which are known to be important in pheromone synthesis (Nation 2001) and a range of other important physiological processes in insects (Canavoso et al. 2001). Moreover, as this study focussed exclusively on males, we still know very little about the effects of macronutrient intake on female fitness. The multidimensional nature of this new framework, coupled with a direct empirical test, is likely to further advance our understanding of the complex interaction between macronutrients and their effects on important fitness-related traits.

2.3 METHODS

Experimental animals

Male and female final instar *Nauphoeta cinerea* nymphs were collected from large cultures of populations known to have high levels of genetic variation and no evidence of inbreeding (Corley et al. 2001). They were placed in single sex cultures with *ad lib* rat chow and water in large test tubes. Each day newly eclosed males and females were randomly allocated to be either focal individuals fed experimental diets, or part of mating assays. Non-focal individuals were stored in individual plastic boxes (11 x 11 x 3 cm) with *ad lib* rat chow food and water to be used only used once in mating assays. Focal individuals were housed in individual plastic containers (17 x 12 x 6 cm) with their diet and *ad lib* water. All animals were housed in a constant temperature room at 28°C with a 14L:10D lighting regime.

Design and construction of artificial diets and measuring intake

A total of 300 artificial, holidic diets were constructed, varying in percentage composition of P, C and L, as well as total nutritional content. Uncorrelated values for P, C and L were generated using a method outlined in Brooks et al. (2005). For each macronutrient in each diet a random number was generated and converted to the inverse of a point on a normal distribution. This was then multiplied by a standard deviation of 75 and added to a mean of 50 to generate the final value. This mean and SD was chosen to provide a wide spread of values encompassing the whole nutritional space. For each diet the sum of P, C and L was between 0 - 96.5% to allow a fixed composition of micronutrients at 3.5%. Any difference between the sum of the macronutrients and 96.5% was made up with cellulose, of which digestion is likely to be minimal in most species, including cockroaches (South et al. 2011). Britannia Finest Beef Dripping (100% animal fat with no added salt or antioxidants) was used as a pure source of lipid. A solid form of lipid was chosen to result in a solid consistency for all diets. Full details on the source of P, C, L and micronutrient mix of the diets is available in the supplementary material (Text S1). Distribution of the diets in the nutritional space is represented in Figure S1.

Experiment 1: No choice of diets

Experimental design and feeding protocol

A single cockroach of each sex were allocated at random to each of the 300 diets on the day they eclosed to adulthood. Diets were dried in an oven at 30°C for 3 days to remove moisture and weighed on an electronic balance (Ohaus Explorer® Pro) to provide each cockroach was provided with approximately 250mg of diet. This amount was sufficient to ensure that cockroaches did not fully consume their diet during each feeding period. Diet was placed in a feeding platform that consists of an upturned vial lid (1.6cm diameter) glued on a petri dish (5.5cm diameter) so that any diet spilt during feeding could be collected. New diet and fresh water was provided every 5 days (a single feeding period) and the old diet was dried at 30°C for 3 days to remove water prior to re-weighing. Diet consumption was calculated for each feeding period by subtracting the final dry weight of diet after feeding from the original dry weight provided to each cockroach. The intake of P, C and L was then calculated from total intake using the percentage composition of the diet.

In total, males received a total of 11 feeds (i.e. 55 days post-eclosion) before being placed an Eppendorf and frozen at -80°C for sex pheromone analysis (see below) and lipid analysis (Chapter 2). Females received feedings until they gave birth to offspring or until they had received 11 feeds. Females were also placed in individual Eppendorfs and frozen at -80°C for lipid analysis (Chapter 2). Nutrient intake was summed across the total number of feeds that each cockroach received. Due to our uneven temporal sampling regime across the sexes, nutrient intake was expressed per day of feeding and this measure was used in all subsequent statistical analyses.

Female mating protocol and fitness measures

After two feeding periods (i.e. 10 days post-eclosion), each experimental female was randomly allocated a 10 day old, virgin male as a mating partner. After diet had been removed, males were added to the empty plastic contained with the female and copulation monitored, with successful copulation being defined as a mating that lasted at least 10 minutes. In the event of successful copulation not occurring, a new male was used as a replacement and this process continued until a successful copulation resulted for all females. Mated males were returned to the large cultures, and females were placed back in their containers with fresh diet and water. For each experimental female we measured gestation time, calculated as the time interval between mating and the birth of offspring, as well as the number of offspring produced. Thirty one females aborted their clutch during the experiment, leaving a total sample size of n = 269 females with measures of nutrient intake, gestation time and offspring number.

Male mating protocol and fitness measures

After two feeding periods (10 days-post eclosion), all focal males were randomly allocated a virgin, 10 day old, virgin female. The mating behaviour of each mating was recorded following the procedure outlined in South et al. (2011) and attractiveness measured as the time interval between a male first raising his wings to initiate courtship until successfully entering copulation, characterized by the male facing 180° from the female (South et al. 2011). All observations were conducted under red light, in individual plastic containers (17 x 12 x 6 cm) with the sides coated

in a thin layer of Vaseline® to prevent the cockroaches from escaping. The male was introduced into this container 5 minutes before the female to allow him to acclimate prior to observing behaviour. The attractiveness of each male was measured in this way for a total of 4 times during the experiment (days 10, 20, 30 and 40 post-eclosion). All females from these matings were housed in individual containers (11 x 11 x 3 cm) and provided *ad libitum* with rat chow and water until they gave birth and the number of offspring were counted. The mean attractiveness and number of offspring produced by females (which we used as a measure of male fertility) across these four mating was used in all subsequent analyses. One male consistently failed to obtain a mating and therefore the total sample size for males with measures of nutrient intake, attractiveness and offspring number was n = 299 males.

The pheromone profile of male *N. cinerea* consists of three individual pheromones: 3-hydroxy-2-butanone (3H2B), 2-methylthiazolidine (2MT) and 4-ethyl-2-methoxyphenol (4E2M). At day 55 post-eclosion, we measured the absolute amount of each of these individual pheromones produced by experimental males using Gas Chromatography-Mass Spectrometry (GC-MS) following the protocol outlined in South et al. (2011). In brief, males were removed from the -80°C freezer and partially defrosted so that the sternum could dissected and blotted on tissue paper to remove any fat. The sternum was submerged in 400μl of HPLC grade dichloromethane containing an internal standard ([E,Z]-4-7-tridecadienyl acetate at 10ng/μl) for 2 hours at room temperature and then removed. Using an autosampler (Agilent CTC PAL), 2 μl of this extract was injected into a DB-Wax column (30m x 0.25 x 0.25μm film thickness) housed in an Agilent 7890 GC coupled with an Agilent 5975 MS, using helium as the carrier gas. The inlet and transfer lines temperatures were set to 200°C and 240°C, respectively and the extract was injected in a pulsed split less mode. After injection of the extract, the column was held at 50°C for 1.5 minutes before being raised at a rate of 10°C/minute to 250°C, with a final hold time of 2 minutes at this temperature. The MS was operated in selected ion mode to limit the output to the three sex pheromones of interest (3H2B, 2MT and 4E2M) with subsequent analysis being limited to the following specific ions: 45, 79, 88, 103, 107, 137 and 152. To quantify the abolsute amounts of the three sex pheromones, samples were quantified against multilevel calibration curves made using pure solutions of the three pheromones at known concentration. 3H2B, 2MT and 4E2M

were purchased in pure form from Sigma-Aldrich (St. Louis, MO), Endeavor Specialty (Daventry, UK) and Pfaltz and Bauer (Watersbury, CT), respectively. Ten males produced very low amounts of all three pheromones (even after running extracts a second time) and were therefore excluded from further analysis. Thus, the total number of males available with measures of nutrient intake and pheromone abundance was *n* = 290 males.

Experiment 2: Choice Experiment

Design of diets

A total of six artificial, holidic diets were produced, varying in protein (P), carbohydrate (C) and lipid (L) content, as well as total nutritional level. Eight dietary treatments were used, each consisting of three diets to form a dietary triplet. Each dietary triplet consisted of one diet with a macronutrient ratio of 1:1:1 (P:C:L), one with a ratio of 1:8:1, and one with a ratio of 1:1:8, ensuring each triplet had one diet where each macronutrient is high relative to the others. Diets also varied across two total nutrition levels, with high nutrition diets having a total nutritional level of 84% (sum of the % content of each macronutrient) and low nutrition diets having a total nutritional level of 36%. Cellulose formed the remainder of the content, alongside a fixed micronutrient content of 3.5%. Table S1 displays the diets used in each triplet, and their nutritional composition.

Experimental design and feeding protocol

A total of 3 cockroaches of each sex were allocated at random to each treatment on the day they eclosed to adulthood. The provisioning and weighing of diet protocol was as in experiment one, with total consumption of each diet calculated, as well as consumption of each of the three macronutrients (P, C, L) using the percentage composition of the diets . Expected random consumption of each of the three macronutrients was also calculated, assuming indiscriminate feeding and equal intake of each available diet. In total, both males and females received 4 feeds (20 days post-eclosion)

Statistical Analysis

As our response variables (i.e. gestation time and offspring number in females, pheromone production, attractiveness and fertility in males) were measured in

different units and the sexes had different absolute nutrient intakes, we standardized these variables to a mean of zero and a standard deviation of one using a Z transformation prior to subsequent analysis. Furthermore, for both female gestation time and our measure of the pre-copulatory attractiveness of male, we reversed the sign of Z transformed value as we expect shorter gestation and courtship times will increase female and female fitness, respectively, and be enhanced with nutrient intake. Consequently, after reversing the sign of these transformed values, large positive values represent females with shorter gestation times and males that are more attractive (i.e. shorter courtship time needed to obtain a mating).

A multivariate response surface approach was used to estimate the linear and nonlinear effects of P, C and L intake on our response variables (Lande & Arnold 1983). This is a hierarchical procedure that first fits a reduced multiple regression model that includes the linear terms for nutrients (P, C, L). This model is used to generate the vector of linear nutritional gradients for each response variable. Next a full multiple regression model that contains the linear, quadratic (P x P, C x C, L x L) and correlational (P x C, P x L, C x L) terms for nutrients. The matrix of quadratic and correlational nutritional gradients (referred to as the **γ**) is derived from this model for each response variable and describes the curvature of the response surface (Lande & Arnold 1983). It is well documented in the selection literature that it is possible to underestimate the strength of the nonlinear effect of nutrients if only the size and significance of the **γ** coefficients are interpretted (Phillips & Arnold 1989; Blows & Brooks 2003). This is because selection is a multivariate process and acts on combinations of traits rather than on individual traits in isolation (Lande & Arnold 1983). The same is expected when considering the effects of multiple nutrients on fitness-related traits, as numerous empirical studies have shown that combinations of nutrients (often viewed as nutrient ratios) are known to optimize these traits (e.g. Lee et al. 2008; Maklakov et al. 2008).

As **γ** is a symmetrical matrix, canonical analysis can be used to re-write the second order regression equation that describes the response surface in a more readily interpreted form (Box & Draper 1987; Phillips & Arnold 1989; Blows & Brooks 2003). This is achieved by matrix diagonalization, a process that translates and rotates the coordinates of the multidimensional space to locate major axes of the

response surface by determining the normalized eigenvectors ($\mathbf{m}_i$) and associated eigenvalues ($\boldsymbol{\lambda}_i$) of $\boldsymbol{\gamma}$. The diagonalization of $\boldsymbol{\gamma}$ results in a matrix Λ:

$$\Lambda = \mathbf{M}^T \boldsymbol{\gamma} \mathbf{M} \quad \text{(Eq.1)}$$

where $\mathbf{M}$ is a matrix that contains the normalized eigenvectors of $\boldsymbol{\gamma}$ as columns. The matrix Λ has the eigenvalues of $\boldsymbol{\gamma}$ along the diagonal and zeros off the diagonal, since $\boldsymbol{\gamma}$ is rotated to remove all cross-product terms:

$$\Lambda = \begin{bmatrix} \lambda_1 & 0 & 0 & 0 \\ 0 & \lambda_2 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & \lambda_i \end{bmatrix} \quad \text{(Eq.2)}$$

The second order regression equation can now be rewritten in canonical form as:

$$\boldsymbol{w} = \alpha + \boldsymbol{y}^T \boldsymbol{\theta} + \boldsymbol{y}^T \Lambda \boldsymbol{y} \quad \text{(Eq.3)}$$

where the individual traits (i.e. nutrient intake in our case) have been replaced by the new major axes (eigenvectors) of the response surface ($\boldsymbol{y}_i$), where a new axis is derived for each trait being examined (i.e. 3 axes in our case). We refer to these major axes ($\mathbf{m}_i$) as nutritional vectors where $\boldsymbol{\theta}_i$ describes the slope of surface along each axis, whereas the eigenvalues ($\boldsymbol{\lambda}_i$) of $\boldsymbol{\gamma}$ describes the strength of the curvature.

For each response variable, we estimated the strength and significance of $\boldsymbol{\theta}_i$ along each nutritional eigenvector using the double regression method of Bisgaard & Ankenman (1996). This approach can inflate type I error rates if used to determine the strength and significance of $\boldsymbol{\lambda}_i$ if only a single set of eigenvectors are derived from the canonical analysis of $\boldsymbol{\gamma}$ (Reynolds et al. 2009). We therefore assessed the strength and significance of $\boldsymbol{\lambda}_i$ using the permutation procedure of Reynolds et al. (2009), which constructs 10,000 unique sets of eigenvectors to account for the error associated with the estimation of $\boldsymbol{\gamma}$ coefficients. These nutritional vectors can be interpreted in the same way as principal components, whereby the contribution of each nutrient to a given nutritional vector is given by its factor loading (presented in the $\mathbf{M}$ matrix). Nonparametric thin-plate splines (Green & Silverman 1994) were used to visualize the major axes of $\boldsymbol{\gamma}$ for each response variable. We refer to these thin-plate splines as the nutritional landscape. We used the Tps function in the FIELDS

package of R (version 2.13.0) to construct the nutritional landscapes for each response variable. In each case, the value of the smoothing parameter that minimised the the generalized cross-validation (GCV) score was used when constructing landscapes and landscapes were plotted in R using a contour view.

A sequential model building approach (Draper & John 1988) was used to determine if the linear and nonlinear effects of nutrient intake differed across response variables and the sexes. Full details of this procedure, as applied to nutrient intake, is provided in South et al. (2011). In short, this approach starts by running a general linear model (GLM) that includes a dummy variable (i.e. type of response variable or sex) as a fixed effect, the linear terms for nutrient intake (P, C and L) as continuous covariates and the standardized variables being compared as the response variable (reduced model). A second GLM is run that includes the interaction terms between the linear terms for nutrient intake and the dummy variable (complete model). In both models, the error sums of squares is recorded, as well as the error degrees of freedom and the number of covariate added in the complete model. These parameters are used in a partial *F*-test to determine if the complete GLM explains significantly more of the variance in the response variables being compared than the reduced GLM (Bowerman & O'Connell 1990). If the inclusion of interaction terms significantly improves the fit of the GLM, this indicates that linear effects of nutrient intake differs across the response variables being compared and inspection of the individual interaction terms for each nutrient from the complete GLM can be used to determine which specific nutrients contribute to this overall significant difference. This process is then repeated by sequentially adding and testing the quadratic effects of nutrients and then the correlational effects of nutrients (South et al. 2011).To determine if male and female cockroaches regulate their intake of nutrients when given dietary choice, a Multivariate Analysis of Variance (MANOVA) was used where diet triplet (1 to 8), sex and their interaction were included as fixed effects and the intake of P, C and L were included as the response variables. As there was a significant interaction between diet triplet and sex (see Table 2.3), we followed this overall model with a separate MANOVA within each sex, as well as univariate ANOVAs to demonstrate how each nutrient contributes to any overall multivariate effect. Within each sex, Fisher's LSD post-hoc tests were conducted for each nutrient to determine which diet triplets differed in mean nutrient

intake. For each diet triplet, we also calculated the intake of P, C and L that would be expected in cockroaches consumed each of the diets in a given triplet at random. This expected value was calculated by (*i*) dividing the total amount of diets consumed in each triple by three, (*ii*) converting this amount to an expected intake of P, C and L for each diet in the triplet by multiplying by the nutrient concentration of the diet and (*iii*) summing the total amount of expected P, C and L ingested on all 3 diets. This expected value of each nutrient intake was subtracted from the actual intake of nutrients for each diet triplet. Positive values therefore mean than individuals consumed more of that nutrient than expected via random feeding, whereas negative values mean that individuals consumed less of that nutrient than expected via random feeding. This difference was analysed using the same MANOVA model outlined above and one-sample *t*-tests were used within each diet triplet to test whether the difference for each nutrient differed from a mean of zero (i.e. indicating that the intake of nutrients did not differ significantly from random feeding).

We calculated the regulated intake point, defined as the point in nutrient space that individuals actively defend when given dietary choice (Simpson & Raubenheimer 2012), for each sex as the mean intake of P, C and L across diet triplets. To determine if nutrient regulation is optimal for the expression of fitness-related traits in the sexes, we mapped the regulated intake point onto the nutritional landscapes for each response variable to visualize the proximity of this point to the peak of the landscape. Prior to mapping, it was necessary to convert the regulated intake point into an **m** score for each axis of the nutritional landscape being examined. This was achieved by substituting the values for the regulated intake point into the equations (i.e. eigenvectors) describing the two major axes of the nutritional landscape being examined for each response variable (provided in Table 2.2).

2.4 RESULTS

Experiment 1: No choice of diets

Female gestation time and offspring number

There were significant linear effects of all three macronutrients on female gestation time, with an increased intake of nutrients resulting in shorter gestation times (Table 2.1A; note: the sign of gestation time reversed for analysis). There were also significant negative quadratic effects of all three nutrients on gestation time, as well as a significant negative correlational gradient for the covariance between P and L (Table 2.1A). Canonical analysis of **γ** for gestation time revealed two nutritional vectors with significant curvature (**m_2** and **m_3**) (Table 2.2A). The dominant nutritional vector (m_3) is heavily loaded to the intake of all three nutrients and **λ** is negative indicating a peak in gestation time along this vector (Table 2.2A). There was also a significant negative slope (***θ***) to this nutritional vector indicating that gestation time decreases as this vector becomes more negative (i.e. the intake of P, C and L are high, Table 2.2A). The second nutritional vector (**m_2**) is heavily loaded to C intake and to a lesser degree P and L and **λ** is also negative indicating a peak in gestation time along this vector (Table 2.2A). Inspection of landscape along these two nutritional vectors shows a clear peak in gestation time (Figure 2.2A) located at negative values of **m_2** (intermediate intake of C) and **m_3** (high intake of all nutrients). There was also significant linear slope on **m_1** which is heavily loaded to P (positively) and L (negatively) (Table 2.2A) and therefore reflects the correlational selection between these nutrients (Table 2.1A).

There was a significant positive linear effect of C intake on the number of offspring produced by a female, as well as large and significant negative quadratic effects of P and C intake (Table 2.1B). Canonical analysis of **γ** for offspring number revealed two nutritional vectors with significant curvature (**m_2** and **m_3**)(Table 2.2B). The dominant vector (**m_3**) was heavily loaded to the intake of C and **λ** is negative indicating a peak in offspring number along this vector (Table 2.2B). There was also a significant negative slope along this vector indicating that offspring number increases with C intake but decreases with P intake (Table 2.2B). The second nutritional vector (**m_2**) is heavily loaded to P and C and **λ** is also negative indicating a peak in offspring number along this vector (Table 2.2B). Inspection of the landscape

along these two nutritional vectors shows a clear peak in offspring number (Figure 2.2B) located at positive values of $\mathbf{m_2}$ (high intake of P and C) and negative $\mathbf{m_3}$ (high intake of C)(Figure 2.2B).

There are significant differences in the linear, quadratic and correlational effects of nutrients in female gestation time and offspring number (Table S2A). The linear difference is due to P and L influencing gestation time but not offspring number, whereas the quadratic difference is driven by the curvature of P being stronger for offspring number than gestation time (Table S2A). The correlational difference is due to the covariance between P and L having a significant effect on gestation time but not offspring number (Table S2A).

Male pheromone production

There were significant positive linear effects of C and L intake, as well as a significant negative linear effect of P intake, on the amount of 3H2B produced by males (Table 2.1C). There were also significant negative quadratic effects of C and L on the amount of 3H2B produced and a significant positive correlational effect on the interaction between C and L intake (Table 2.1C). Canonical analysis of $\boldsymbol{\gamma}$ revealed two nutritional vectors with significant curvature ($\mathbf{m_2}$ and $\mathbf{m_3}$) (Table 2.2C). The dominant nutritional vector ($\mathbf{m_3}$) was heavily loaded to the intake of C (positive) and L (negative) and $\boldsymbol{\lambda}$ was negative indicating a peak in the production of 3H2B along this nutritional vector. The second nutritional vector ($\mathbf{m_2}$) was also heavily loaded to C and L intake (both negative) and $\boldsymbol{\lambda}$ was also negative indicating a peak in the production of 3H2B along this vector (Table 2.2C). There was also a significant negative linear slope to this nutritional vector favouring increases in the intake of P and L (Table 2.2C). Visualization of the nutritional landscape (Figure 2.3A) along these two nutritional vectors shows a peak in 3H2B at negative values of $\mathbf{m_2}$ (high intake of C and L) and values of $\mathbf{m_3}$ centred close to zero. There was also a significant negative slope along $\mathbf{m_1}$ which is positively loaded to P intake which means this vector represents a decreased intake of P (Table 2.2C).

There were significant positive linear effects of C and L intake, as well as a significant negative quadratic effect of L, on the amount of 2MT produced by males (Table 2.1D). Canonical analysis of $\boldsymbol{\gamma}$ revealed two nutritional vectors with significant curvature ($\mathbf{m_2}$ and $\mathbf{m_3}$) (Table 2.2D). The dominant nutritional vector ($\mathbf{m_3}$) was

heavily loaded to the intake of L and to a lesser degree the intake of C (both positive) and **λ** was negative indicating a peak in the production of 2MT along this nutritional vector. There was also a significant positive linear slope to this vector favouring a higher intake of these two nutrients (Table 2.2D). The second nutritional vector (**m_2**) was heavily loaded L intake (positive) and **λ** was also negative indicating a peak in the production of 2MT along this vector (Table 2.2D). Visualization of the nutritional landscape along these two nutritional vectors (Figure 2.3B) shows a peak in 2MT production at positive values of **m_2** (high intake of L) and **m_3** (high intake of C and L).

There were significant positive linear effects of C and L, as well as significant negative quadratic effects of all nutrients, on the amount of 4E2M produced by males (Table 2.1E). Canonical analysis of **γ** revealed two nutritional vectors with significant curvature (**m_2** and **m_3**) (Table 2.2E). The dominant nutritional vector (**m_3**) was heavily loaded to the intake of C and L (both positive) and **λ** was negative indicating a peak in the production of 2MT along this nutritional vector. There was also a significant positive linear slope to this vector favouring a higher intake of these two nutrients (Table 2.2E). The second nutritional vector (**m_2**) was heavily loaded L intake (positive) and **λ** was also negative indicating a peak in the production of 2MT along this vector (Table 2.2E). Visualization of the nutritional landscape along these two nutritional vectors (Figure 2.3C) shows a peak in 4E2M production at values of **m_2** approaching zero and at positive values of **m_3** (high intake of C and L). There was also a significant linear slope along **m_1**, which favours an increased intake of C and L and a decreased intake of P (Table 2.2E).

Pairwise comparisons of the linear, quadratic and correlational effects of nutrient intake on the production of the three male pheromone components showed that 3H2B differed significantly from 2MT in the correlational effects of nutrient intake (Table S2B) and from 4E2M in the linear effects of nutrient intake (Table S2C). The difference in correlational effects was driven by a significant positive correlational gradient between C and L intake for 3H2B production but not for 2MT production, whereas the difference in linear effects was due to a significant negative linear effect of P intake in 3H2B production but not for 4E2M production (Table S2C). There were no differences in the linear, quadratic or correlational effects of nutrient intake between 2MT and 4E2M (Table S2D)

Male attractiveness

There were significant positive linear effects of C and L intake, as well as significant negative linear effects of P intake, on the pre-copulatory attractiveness of males (Table 2.1F). There were also significant negative quadratic effects of C and L intake, as well as a significant positive correlational effect on the interaction between C and L intake (Table 2.1F). Canonical analysis of **γ** revealed two nutritional vectors with significant curvature ($\mathbf{m_2}$ and $\mathbf{m_3}$) (Table 2.2F). The dominant nutritional vector ($\mathbf{m_3}$) was heavily loaded to the intake of C and L (both negative) and **λ** was negative indicating a peak in male attractiveness along this nutritional vector. There was also a significant negative linear slope to this vector meaning that a higher intake of these two nutrients increased male attractiveness (Table 2.2F). The second nutritional vector ($\mathbf{m_2}$) was heavily loaded to all three nutrients (negative to P and C intake and positive to L intake) and **λ** was also negative indicating a peak in male attractiveness along this vector (Table 2.2F). There was also a significant positive linear slope for this nutritional vector favouring an increase in male attractiveness with L intake (Table 2.2F). Visualization of the nutritional landscape along these two nutritional vectors (Figure 2.4A) shows a peak in male attractiveness at positive values of $\mathbf{m_2}$ (increased L intake) and at negative values of $\mathbf{m_3}$ (increased intake of C and L). There was also a significant negative linear slope along $\mathbf{m_1}$, which favours an increased intake of C and a decreased intake of P (Table 2.2F).

There was no significant difference in the linear, quadratic or correlational effects of nutrient intake on male attractiveness and 3H2B (Table S2E) or 2MT (Table S2F) production. There was, however, a significant difference in the linear effects of nutrients on male attractiveness and the production of 4E2M due to the fact that P intake decreases male attractiveness but does not influence the production of 4E2M and because the production of 4E2M is more responsive to the intake of C than male attractiveness (Table S2G).

Male fertility

There were significant positive linear effects of P and C intake on the fertility of males (Table 2.1G). There were also significant negative quadratic effects of P and C intake, as well as a significant negative correlational effect on the interaction between C and L intake (Table 2.1G). Canonical analysis of **γ** revealed two

nutritional vectors with significant curvature ($\mathbf{m_2}$ and $\mathbf{m_3}$) (Table 2.2G). The dominant nutritional vector ($\mathbf{m_3}$) was heavily loaded to the intake of C and L (both positive) and $\boldsymbol{\lambda}$ was negative indicating a peak in male fertility along this nutritional vector. There was also a significant positive linear slope to this vector meaning that a higher intake of these two nutrients increased male fertility (Table 2.2G). The second nutritional vector ($\mathbf{m_2}$) was heavily loaded to P intake (positive) and $\boldsymbol{\lambda}$ was also negative indicating a peak in male attractiveness along this vector (Table 2.2G). There was also a significant positive linear slope for this nutritional vector favouring an increase in male fertility with P intake (Table 2.2G). Visualization of the nutritional landscape along these two nutritional vectors (Figure 2.4B) shows a peak in male fertility at positive values of $\mathbf{m_2}$ (increased C and L intake) and $\mathbf{m_3}$ (increased intake of P). There was also a significant negative linear slope along $\mathbf{m_1}$, which favours an increased intake of C and a decreased intake of L (Table 2.2G).

There were significant differences in the linear, quadratic and correlational effects of nutrient intake on male attractiveness and fertility (Table S2H). The difference in linear effects were due to the fact that male fertility increased with P intake but male attractiveness did not and that male attractiveness increased with L intake but male fertility did not (Table S2H). The difference in quadratic effects is due to their being a significant negative quadratic term for L intake on male attractiveness but not for male fertility (Table S2H). The difference in correlational effects is due to the fact that the effect of the covariance between C and L intake is positive for male attractiveness but negative for male fertility (Table S2H).

Comparison of nutritional effects across the sexes

We compared the effects of nutrient intake on female offspring production to those for male pre-copulatory attractiveness and fertility (Table S3). There were significant differences in the linear and quadratic (but not correlational) effects of nutrient intake on female offspring production and male attractiveness (Table S3A). The difference in linear effects was due to the fact that female offspring production was significantly more responsive to the intake of C than male attractiveness (Table S3A). The difference in quadratic effects was due to the fact that there is a significant negative quadratic for P intake on female offspring production but not for male attractiveness

and because the curvature for C intake is stronger for female offspring production than male attractiveness (Table S3A).

There was a significant difference in the quadratic effects of nutrients on female offspring production and male fertility, but not for the linear or correlational effects (Table S3B). This difference is due to the curvature for P and C intake on female offspring production being significantly stronger than for male fertility (Table S3B).

Experiment 2: Nutrient intake under dietary choice

MANOVA revealed significant differences in the mean intake of nutrients across diet triplets, the sexes and there was also a significant interaction between diet triplet and sex (Table 2.3). Across diet triplets females consumed, on average, 50% more of each nutrient than males (Figure 2.5). Within each sex, there was significant variation in the intake of P, C and L across diet triplets (Table 2. 3, Figure 2.5). In females, there was a significantly higher intake of C than P and L in 6 out of 8 diet triplets (the exceptions being diet triplets 4 and 7)(Figure 2.5). In males, there was a significantly higher intake of C than P and L in 7 out of 8 diet triplets (the exception being diet triplet 7)(Figure 2.5). The extent to which C intake exceeds P and L intake across diet triplets and the sexes, accounts for the diet triplet by sex interaction (Table 2.3). Not surprisingly, a MANOVA showed a significant effect of diet triplet, sex and their interaction on the difference in nutrient intake from that expected under random feeding on diets. In both sexes, individuals consistently consumed more C than expected under random feeding across diet triplets (Figure S2). In most cases, they also consumed significantly less P and L than expected, although this varied across diet triplets (e.g. diet triplets 3 and 4)(Figure S2).

Despite this variation in nutrient intake across the sexes, the regulated intake point was remarkably similar for the sexes, estimated at a P:C:L ratio of 1:3.83:1.47 in females and 1:3.84:1.51 in males. The regulated intake points (±SEs) are mapped onto the nutritional landscapes for each of our response variables (white crosses) for females (Figure 2.2) and males (Figures 2.3 and 2.4). In females, the regulated intake point was in close proximity to the optimum for both gestation time (Figure 2.2A) and offspring production (Figure 2.2B), whereas this point was located further from the optimum for male pheromone expression (Figure 2.3A-C), pre-copulatory

attractiveness (Figure 2.4A) and fertility (Figure 2.4B). This suggests that females are better at optimally regulating their intake of nutrients than males in *N. cinerea*.

2.5 DISCUSSION

Here, I have introduced a relatively simple extension to the NGF to increase its dimensionality so that the effects of more than two nutrients and total nutrition can be examined simultaneously. Furthermore I have applied this framework to examine the role of three macronutrients (P, C and L) on important fitness-related traits in male and female cockroaches (*Nauphoeta cinerea*) to demonstrate that it is the balanced intake of these nutrients rather than the intake of calories *per se*, that maximise these important fitness-related traits. Moreover, when cockroaches are given dietary choice between three different diets, the sexes regulate their intake of nutrients in a similar way, having almost identical regulated intake points. This regulated intake point is closer to the optima for traits in females than males, suggesting that the former is better able to regulate their intake of nutrients to maximise fitness.

Gestation time was shortest at high intake of all three macronutrients (Figure 2.2, Table 2.2) with the dominant nutritional vector ($\mathbf{m}_3$) heavily loaded to the intake of all three nutrients. This could be due to female control over gestation, or energy sources for developing embryos. In cockroaches a possible benefit of short gestation time is that the resultant offspring then reach sexually maturity faster (Moore, 1994), although this may be countered by the fact that female lifespan decreases with shorter gestation times (Moore et al. 2003). There may be further benefits to females if carrying offspring is costly to mobility, although this varies between taxa (Plaut 2002; Miettinen et al. 2006), or shorter gestation allows them to reproduce again sooner.

Female offspring number was significantly affected by carbohydrate and protein intake (Figure 2.2, Table 2.2), with C intake of greater importance than P intake in maximising offspring number along the two significant nutritional vectors $\mathbf{m}_2$ and $\mathbf{m}_3$. P intake intake is often of paramount importance for female fecundity (Hamilton et al. 1990; Barry et al. 2010). L intake can also be important for fecundity

(Ziegler, 2006; Jensen et al. 2012), although that wasn't the case here. The importance of C intake for female offspring number may reflect a role in egg development and metabolism (Bauerfeind & Fischer, 2005).

The macronutrient balance that maximises gestation time differs significantly from that which maximises offspring number (Table S2A). These differences are unrelated to offspring size, which is not affected by macronutrient intake in this species. Gestation is more responsive to protein and lipids on a linear scale, but there are large non-linear effects of P on offspring number, which are stronger than that for gestation time. There are also differences in correlational effects, with high levels of both C and L shortening gestation time, but leading to decreases in offspring number, possibly due to the propensity for females to store C and L in their fat bodies (Chapter 2) as a possible insurance against future food shortage (Goenaga et al. 2013) rather than invest in maximising immediate reproduction.

Male sex pheromone expression was strongly influenced by the balance of macronutrients consumed (Figure 2.3, Table 2.2). Previous work has emphasized the role of C intake in the pheromone expression of insects (South et al. 2011; Fedina et al. 2012), but here L intake had greater effects on two of the pheromones (3H2B & 4E2M) with C intake also of high importance in the dominant nutritional vectors and peaks for each pheromone. This is unsurprising given that pheromones have lipid precursors, and cockroaches store carbohydrates as lipids (Nation, 2001). Male pre-copulatory attractiveness was also significantly affected by all three macronutrients (P, C and L) (Figure 2.4, Table 2.2), with high P intake decreasing attractiveness and high C and L intake increasing attractiveness through linear, quadratic and correlational effects (Figure 2.4) as well as through heavy loading of the two significant nutritional vectors ($\mathbf{m_2}$ and $\mathbf{m_3}$). Male fertility, as measured by the number of offspring four female mating partners fed standard diets produced, was significantly affected by C and particularly P intake, with L being of less importance (Figure 2.4, Table 2.2).

These three major components of male reproductive fitness are all strongly influenced by macronutrient intake, but in different ways. In *N. cinerea*, attractiveness is strongly linked to a male's sex pheromone profile (South et al. 2011), with female fecundity and gestation time both effected by pheromone

exposure from their mating partners (Moore et al. 2001, 2003). There are however some differences in macronutrient balance that maximise pheromone production and pre-copulatory attractiveness, with lipids of marginally greater importance for pheromone expression, and carbohydrates for maximising pre-copulatory attractiveness (TableS2). This may reflect a role of energetic ability in courtship (Moore & Breed, 1986) requiring substantial carbohydrate reserves as well as optimal pheromone profiles to maximise attractiveness. This study may also have underestimated this component of pre-copulatory attractiveness, given that we measured attractiveness in one-on-one interactions where males don't have to compete for access to the female.

Male fertility was maximised at a quite different space on the nutritional landscape (Figure 2.4), with high protein intake being a primary determinant of optimal fertility, but having minimal effects on pheromone profiles or attractiveness. This measure of fertility may be partly influenced by females through differential allocation of resources in response to attractiveness (Evans et al. 2010; Skinner & Watt, 2007), but direct effects in males are likely to be particularly important, especially those involving sperm. A recent study found male fertility to peak at a very similar P:C ratio to sperm number (Bunning et al. 2015), which would explain the large effect of protein on fertility in this study given the known relationship between protein intake and sperm transfer in insects (Gage & Cook 1994; Blay & Yuval 1997). The fact that *N. cinerea* use sperm over multiple reproductive events suggests sperm competition intensity is likely to be low, and males are selected to produce large spermataphores to prevent re-mating rather than produce competitive sperm (Moore et al. 2003). Indeed Bunning et al. (2015) found no effect of diet on sperm viability, but a large effect on sperm number. The same study found evidence of a trade-off between pre- and post-copulatory traits, which is corroborated here with the nutritional differences in maximising fertility and attractiveness. The added resolution gained by studying energy dense lipids (Arrese & Soulages, 2010) as well as protein and carbohydrate allows new insight to be gained in the role of nutrition in these trade-offs. C and L are often combined into one factor due to restrictions of the current framework, but this may hide important effects.

As well as differences within the sexes there were also substantial differences between the sexes. Female fitness traits were maximised at a higher ratio of C to L

than male traits, and were generally more responsive to C intake than male traits. These differences could be partly attributable to the short term measure of female fitness, but lifetime fecundity is often maximised at lower P intake than one-off fecundity (Lee et al. 2008) with C intake having little effect. There are likely to be various factors which contribute to this difference; the sexes likely have different nutritional needs due to differing strategies for maximising fitness, resulting in different consequences of macronutrient intake (Hunt et al. 2004; Adler et al. 2013).

Here, however, I found that males and females share a highly similar regulated intake point, with the regulated intake ratio (P:C:L) being 1:3.83:1.47 in females and 1:3.84:1.51 in males. There were, however, some differences in preference in certain dietary combinations (Figure 2.5). The slight differences in regulated intake point observed here may represent noise from a limited sample size, or slight variation in regulation. The fact that females tend to eat 50% more drives the significant effect of sex on regulation, with both sexes tending to eat more C than expected from indiscriminate feeding, and less of L and P (Figure S2). Variation in C intake seems to largely drive the interaction between sex and treatment, with effect sizes being much smaller for P and L. The differing uses of C for the sexes, with males using C to eventually produce pheromones (Nation, 2001) and females storing C as fat may explain these slight differences. Further to this, triplets 4 and 7 seem to vary most between males and females (Figure 2.5). These represent C biased diets low in nutrition, and males and females may have different life-history responses to this scenario (Valle et al. 2005).

Males and females sharing their regulated intake point is uncommon in the species tested (Maklakov et al. 2008; Harrison et al. 2014) where some divergence usually occurs, and may reflect constraints in reaching their fitness optima, which are often markedly different (Morehouse et al. 2010; Reddiex et al. 2013). In crickets optimal diets were substantially different for males and females, and preferences were only partially diverged towards respective optima, suggesting sexual conflict (Maklakov et al. 2008). It is important however to note that choice at the point of intake is not the only way males and females can adaptively diverge their use of nutrients. There can be sex differences in post-ingestive regulation (Telang et al. 2003; Lee, 2010), which can evolve in response to diet (Riha & Luckinbill, 1996; Warbrick-Smith et al. 2006), thus differing selection on males and females could

have resulted in divergent adaptive changes in post-ingestive regulation that may partially alleviate conflict.

Previous work in *N. cinerea* using two macronutrients (P and C) found that male intake is regulated to a ratio that is the intermediate between those that maximise fertility and pre-copulatory attractiveness (Bunning et al. 2015). In this study further complexities emerge, with regulation not at an intermediate between fertility and attractiveness at the scale of three macronutrients. In contrast the regulated intake point was a much closer match to the optima for the female reproductive traits of gestation time and offspring number. It is important however to note that I have only measured female offspring production in one reproductive event. Female *Drosophila* chose a diet intermediate between maximising lifespan and egg production rate to maximises lifetime fecundity (Lee et al. 2008). This may shift the true peak of female fecundity slightly away from the regulated intake point, however it remains clear that the shared intake regulation is much more optimal for females than it is for males, raising the prospect of females being better at regulating their intake than males. Previous work that has found near shared regulation between the sexes found either equal displacement between the sexes (Reddiex et al. 2013) or that females were particularly pulled away from their optima (Maklakov et al. 2008). In *Drosophila,* males and females mostly share the underlying genetic basis of diet regulation (Reddiex et al. 2013) with directional selection acting on both sexes. Given the likelihood of sex differences in post ingestive regulation (Lee 2010) further work is required to estimate the net result of regulation, and how this matches with optima, to determine whether females still appear to be behaving much more optimally than males.

The extended framework presented here has widespread applications, with ageing research one area that could particularly benefit. The potential for calorie restriction (CR) to be a universal mechanism for delayed ageing is a pervasive and well-studied aspect of gerontology (Kennedy et al. 2007; Nagakawa et al. 2012). In recent years work has begun on testing these effects on primates, but results so far are conflicting (Mattison et al. 2012; Colman et al. 2009, 2014). Methodological and statistical frameworks need to be rigid to allow strong and comparable conclusions to be drawn from manipulations. This extension of the NGF provides the framework to apply a consistent methodology to the effects of dietary restriction on lifespan.

Recent work beginning to investigate three macronutrients simultaneously in mice found that a balance of macronutrients is more important than calorie intake in ageing (Solon-Biet et al. 2014). If this is true of primates as well as rodents it could have strong implications for the use of dietary restriction to combat ageing. The NGF allows further insight into the mechanisms of these effects through mapping of candidate factors such as nutrient signalling pathways TOR and IGF (Partridge et al. 2011) onto landscapes of macronutrient intake and lifespan.

Extending the NGF to three dimensions allows its usefulness to be extended in the field of applied nutrition. Aquaculture has embraced the NGF as a way of optimizing commercial feeds (Ruohonen et al. 2007; Zhang et al. 2012) and the NGF is also beginning to be used to optimize feed for different livestock (Cowieson et al. 2014). Mixture triangles are often used for this, and they have also proven useful in studying nutritional choice in the field (Johnson et al. 2013) and opening new taxa to the NGF (Raubenheimer et al. 2015). The extended framework outlined here is complementary to mixture triangles. Understanding the effects of total nutrition, which mixture triangles miss, can improve the reliability of the conclusions drawn from mixture triangle studies. Studies using mixture triangles (Solon-Biet et al. 2014) can also provide insight into traits such as ageing and obesity that the new framework can than investigate further.

Macronutrients themselves are composed of constituent parts. Individual amino acids have been implicated in the effects of protein restriction on lifespan extension (Grandison et al. 2009), and this framework allows the possibility of examining these effects in a realistic context with simultaneous manipulations. Micronutrients can also be considered, many of which can have important independent fitness consequences (Mand & Tilgar, 2003; Ayala et al. 2006). It is not known to what extent intake of micronutrients is correlated with intake of other macronutrients, or whether they are regulated independently. This framework offers the potential to incorporate micronutrients into a fully integrative framework alongside macronutrients.

Over the past several decades understanding of the role of nutrition has changed, and there is now a widespread appreciation of the importance of the balance of macronutrients, rather than amounts of one macronutrient or total energy

intake. The new framework outlined here builds on the benefits of the NGF and alleviates some of the limitations, making it more nutritionally relevant and applicable to the emerging questions in the field. I have demonstrated the potential of the framework to broaden understanding of the role of nutrition in fitness traits in insects, and it has uses far beyond that in every area where nutritional geometry is used. The simple nature of manipulations, combined with the ability to map any fitness trait against these manipulations, provide a solid and widely applicable framework for understanding nutrition.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 2.1. Protein and carbohydrate composition of the 24 experimental diets commonly used in nutritional geometry. The 6 nutritional rails on which the diets are placed are shown as solid lines. Relative caloric content of the diets is shown using the dashed isocaloric lines.

Figure 2.2. Nutritional landscape along the two major nutritional vectors ($\mathbf{m_2}$ and $\mathbf{m_3}$) for female (A) gestation time and (B) offspring production. The regulated intake point (±SE) determined from Experiment 2 is provided in the white bars. It is important to note that the sign of gestation time is reversed (see text for more details) so that red regions mean females with shorter gestation times.

Figure 2.3. Nutritional landscape along the two major nutritional vectors ($\mathbf{m_2}$ and $\mathbf{m_3}$) for male pheromone production which consists of three pheromone components (A) 3H2B (2) 2MT and (C) 4E2M (see text for more details). The regulated intake point (±SE) determined from Experiment 2 is provided in the white bars.

Figure 2.4. Nutritional landscape along the two major nutritional vectors ($\mathbf{m_2}$ and $\mathbf{m_3}$) for male (A) pre-copulatory attractiveness and (B) fertility. It is important to note that the sign of male attractiveness is reverse (see text for more details) so that red regions represent males with faster courtship times and higher attractiveness scores. The regulated intake point (±SE) determined from Experiment 2 is provided in the white bars.

Figure 2.5. The mean (±SE) intake of protein (black bars), carbohydrate (grey bars) and lipid (white bars) across diet triplets for (A) female and (B) male *N. cinerea*. Bars with different letters are significantly different at $P < 0.05$ using Fisher's LSD post-doc analysis.

Figure 2.1

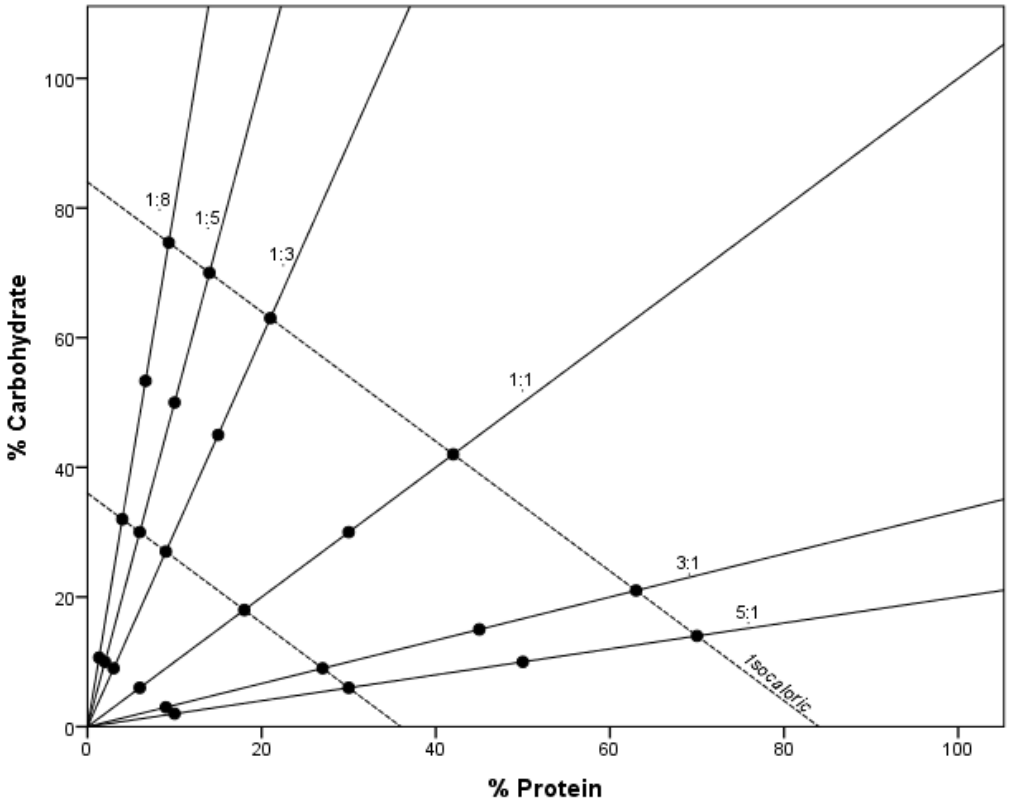
% Carbohydrate
% Protein
0
20
40
60
80
100
1:8
1:5
1:3
1:1
3:1
5:1
isocaloric

Figure 2.2

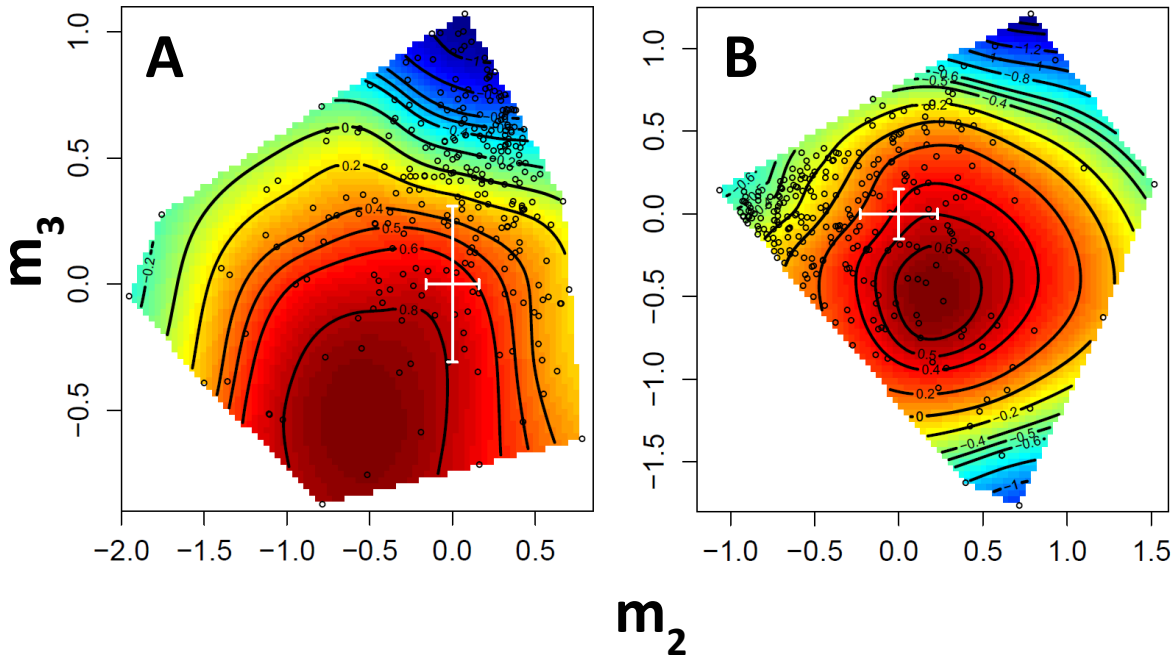

Figure 2.3

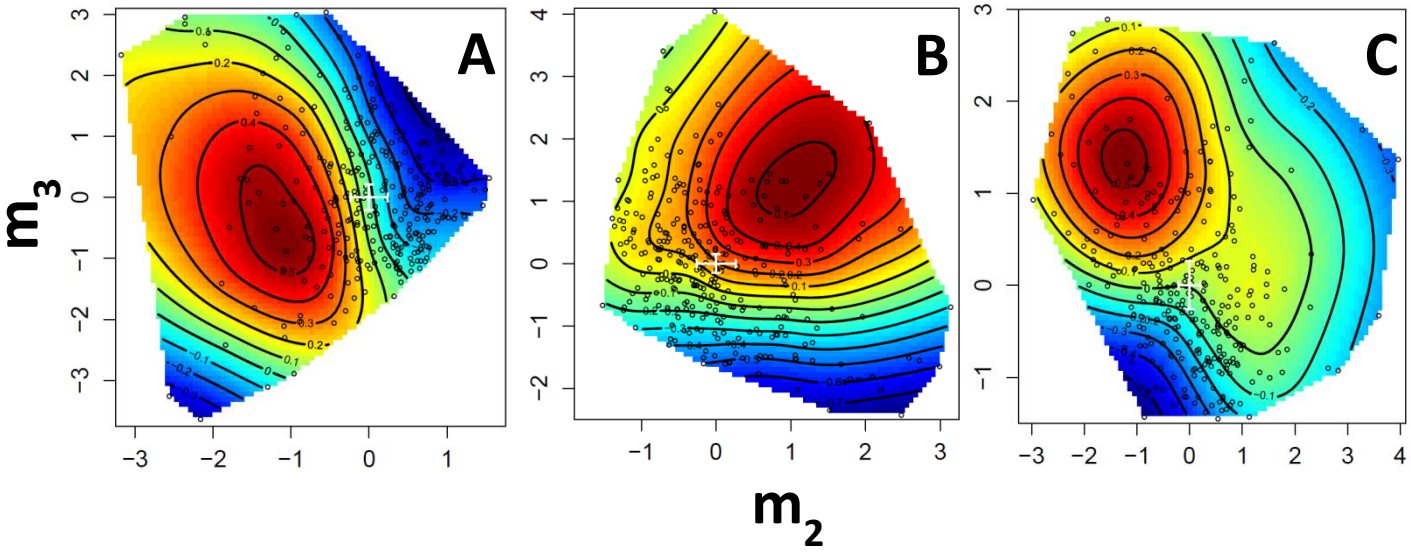

Figure 2.4

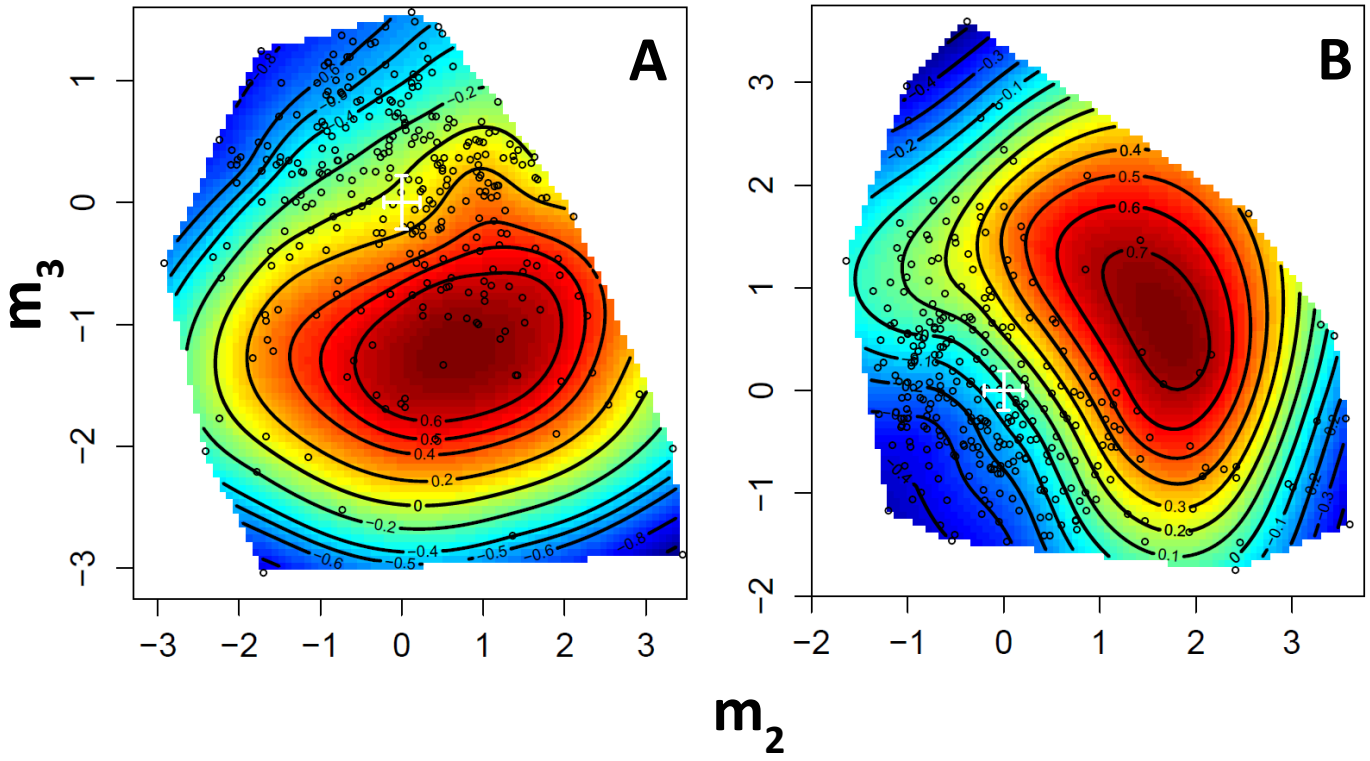

Figure 2.5

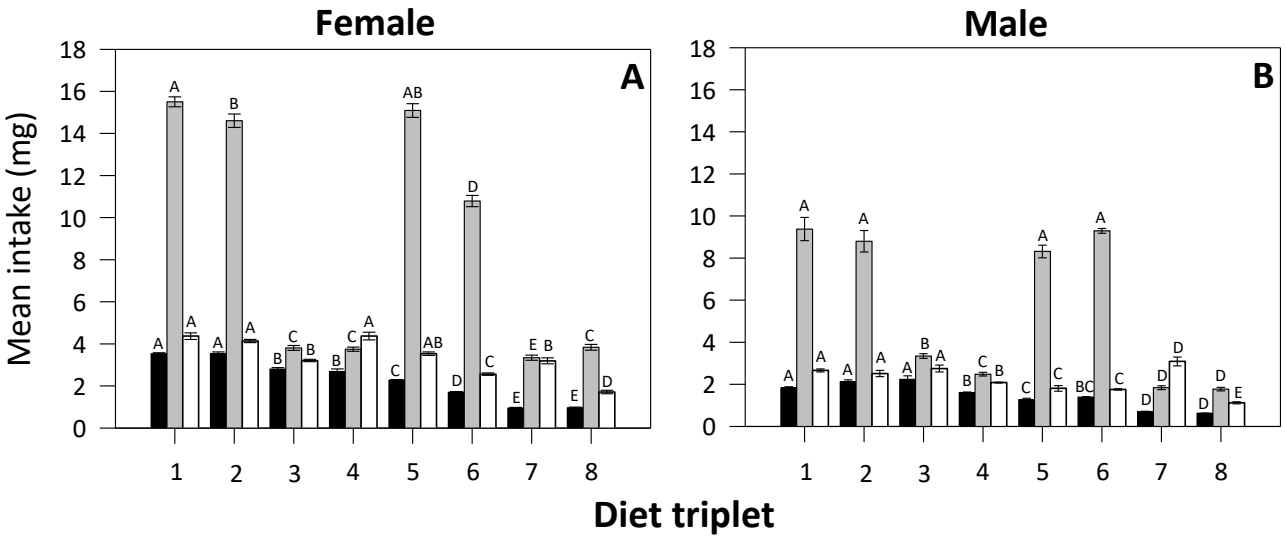
Female
Male
A
B
Mean intake (mg)
Diet triplet
0
2
4
6
8
10
12
14
16
18
1
2
3
4
5
6
7
8

TABLE LEGENDS

Table 2.1. The linear and nonlinear coefficients (±SE) of protein (P), carbohydrate (C) and lipid (L) intake on fitness-related traits in (A) female and (B) male *N. cinerea*. In **γ**, quadratic terms are given along the diagonal (P x P, C x C and L x L) and correlational terms (P x C, P x L and C x L) in the off-diagonal position. Significance test: * $P < 0.05$, ** $P < 0.001$, ** $P < 0.0001$.

Table 2.2. The **M** matrix of eigenvectors ($\mathbf{m_1}$ to $\mathbf{m_3}$) from the canonical analysis of **γ** for fitness-related traits in (A) female and (B) male *N. cinerea*. The linear ($\boldsymbol{\theta}_i$) and quadratic ($\boldsymbol{\lambda}_i$) terms describe the slope and curvature of a given nutritional vector, respectively, and are provided in the last two columns. Significance test: * $P < 0.05$, ** $P < 0.001$, ** $P < 0.0001$.

Table 2.3. Multivariate Analysis of Variance (MANOVA) examining differences in the intake of protein (P), carbohydrates (C) and lipid (L) across diet triplets and the sexes when individuals are provided with dietary choice. As there were differences in the intake of these nutrients across the sexes, we followed this complete model with univariate Analysis of Variance (ANOVA) within the sexes to determine how specific nutrients contributed to this overall multivariate effect.

Table 2.1.

		Γ		
Nutrient intake	Linear Effects	P	C	L
Females				
A. Gestation time				
P	0.93 ± 0.13 ***	-0.47 ± 0.22*		
C	0.66 ± 0.12***	-0.49 ± 0.30	-0.63 ± 0.19**	
L	0.45 ± 0.14 **	-0.94 ± 0.38*	-0.32 ± 0.31	-0.45 ± 0.22*
B. Offspring number				
P	0.02 ± 0.15	-0.92 ± 0.23***		
C	0.39 ± 0.13**	0.46 ± 0.32	-0.96 ± 0.20***	
L	0.01 ± 0.15	-0.09 ± 0.41	0.48 ± 0.33	0.15 ± 0.23
Males				
C. 3H2B				
P	-0.20 ± 0.06 **	0.04 ± 0.04		
C	0.15 ± 0.06*	-0.02 ± 0.08	-0.09 ± 0.04*	
L	0.22 ± 0.06***	-0.06 ± 0.06	0.14 ± 0.07*	-0.08 ± 0.04*
D. 2MT				
P	-0.10 ± 0.06	-0.05 ± 0.04		
C	0.15 ± 0.06*	-0.06 ± 0.09	0.01 ± 0.04	
L	0.15 ± 0.06 *	0.09 ± 0.06	-0.12 ± 0.08	-0.10 ± 0.04*
E. 4E2M				
P	-0.01 ± 0.06	-0.11 ± 0.04*		
C	0.30 ± 0.06***	-0.06 ± 0.08	-0.12 ± 0.04**	
L	0.20 ± 0.06**	-0.07 ± 0.06	0.05 ± 0.08	-0.10 ± 0.04*
F. Attractiveness				
P	-0.19 ± 0.06**	-0.05 ± 0.04		
C	0.13 ± 0.06*	-0.09 ± 0.08	-0.12 ± 0.04**	
L	0.22 ± 0.06***	-0.01 ± 0.05	0.15 ± 0.07*	-0.17 ± 0.04***
G. Fertility				
P	0.17 ± 0.06**	-0.12 ± 0.04**		
C	0.18 ± 0.06**	0.04 ± 0.08	-0.08 ± 0.04*	
L	0.02 ± 0.06	-0.05 ± 0.06	-0.26 ± 0.08**	-0.03 ± 0.04

Table 2.2.

	M				Nutritional effects	
	P	C	L		θ_i	λ_i
Females						
A. Gestation time						
$\mathbf{m_1}$	0.72	-0.10	-0.69		0.29**	0.04
$\mathbf{m_2}$	0.30	-0.85	0.43		-0.09	-0.92**
$\mathbf{m_3}$	-0.63	-0.52	-0.58		-1.19***	-2.20***
B. Offspring number						
$\mathbf{m_1}$	0.00	0.20	0.98		0.09	0.39
$\mathbf{m_2}$	0.77	0.62	-0.13		0.26	-1.46**
$\mathbf{m_3}$	0.24	-0.76	0.16		-0.28*	-2.42***
Males						
C. 3H2B						
$\mathbf{m_1}$	0.93	-0.21	-0.31		-0.29***	0.10
$\mathbf{m_2}$	-0.36	-0.68	-0.64		-0.17*	-0.06*
$\mathbf{m_3}$	-0.08	0.70	-0.71		-0.04	-0.30***
D. 2MT						
$\mathbf{m_1}$	0.39	-0.82	0.43		-0.10	0.11
$\mathbf{m_2}$	0.20	0.28	0.84		0.02	-0.12*
$\mathbf{m_3}$	-0.37	0.50	0.88		0.22***	-0.27***
E. 4E2M						
$\mathbf{m_1}$	0.62	-0.48	-0.63		-0.27***	-0.10
$\mathbf{m_2}$	0.38	-0.42	0.77		-0.00	-0.27***
$\mathbf{m_3}$	0.14	0.71	0.69		0.23**	-0.28*
F. Attractiveness						
$\mathbf{m_1}$	0.78	-0.57	0.26		-0.16**	-0.04
$\mathbf{m_2}$	-0.59	-0.55	0.59		0.17**	-0.18**
$\mathbf{m_3}$	-0.20	-0.62	-0.76		-0.21**	-0.45***
G. Fertility						
$\mathbf{m_1}$	-0.16	-0.63	0.76		-0.13*	0.16
$\mathbf{m_2}$	0.99	-0.09	0.13		0.15*	-0.25**
$\mathbf{m_3}$	-0.01	0.77	0.64		0.15*	-0.37**

Table 2.3.

	MANOVA					Univariate ANOVAs			
Source	Pillai's Trace	*F*	*df*	*P*		Nutrient	*F*	*df*	*P*
Diet triplet (A)	2.91	152.69	21,96	0.0001		P	256.26	7,47	0.0001
						C	608.23	7,47	0.0001
						L	71.63	7,47	0.0001
Sex (B)	0.96	222.23	3,30	0.0001		P	564.07	1,47	0.0001
						C	590.07	1,47	0.0001
						L	392.26	1,47	0.0001
A x B	2.55	26.08	21,96	0.0001		P	30.75	7,47	0.0001
						C	48.25	7,47	0.0001
						L	21.59	7,47	0.0001
Females									
Diet triplet	2.94	114.56	21,48	0.0001		P	273.18	7,23	0.0001
						C	664.65	7,23	0.0001
						L	67.30	7,23	0.0001
Males									
Diet triplet	2.89	60.93	21,48	0.0001		P	59.66	7,23	0.0001
						C	143.33	7,23	0.0001
						L	28.46	7,23	0.0001

3. A MULTIVARIATE VIEW OF MACRONUTRIENT BALANCE AND OBESITY IN THE COCKROACH *NAUPHOETA CINEREA*

3.1 ABSTRACT

Obesity is a central issue in nutritional research, leading to substantial adverse effects in metabolic diseases and disorders. Current evidence suggests that diet consumption is the primary driver of obesity, making understanding the role of diet of paramount importance in tackling the consequences of obesity. Present research in this field has mainly focussed on the effects of single macronutrients or total caloric intake, despite evidence that macronutrient balance may actually be more important. Here, we use recent advances to the Nutritional Geometric Framework to assess the effects of individual macronutrients as well as macronutrient balance on lipid deposition in male and female cockroaches (*Nauphoeta cinerea)*. All three macronutrients (protein, carbohydrate, lipids) had independent and combined effects in lipid deposition in males and females. However, the effect of maconutrient intake on lipid deposition differed significantly across the sexes , with lipid deposition largely increasing with carbohydrate intake in males and largely through lipid intake in females. Despite this difference, my previous work (Chapter 1) has shown that males and females regulate their intake of macronutrients to the same P:C:L ratio when given dietary choice. This multivariate approach has great potential in studying and combatting obesity, by aiding our understanding of how individuals balance their intake of multiple macronutrients.

Keywords: Obesity, Nutritional Geometry, *Nauphoeta cinerea*, multivariate selection analysis, lipid deposition

3.2 INTRODUCTION

Obesity (conventionally defined as a BMI ≥30 kg/m^2) is increasing globally (Ng et al. 2014), having more than doubled worldwide since 1980 (Finucane et al. 2011), and is a key topic in nutritional research. Despite some evidence of the increase plateauing (Ng et al. 2014) there are forecast to be huge global increases in obesity in the coming decades (Kelly et al. 2008). Obesity has substantial negative consequences, particularly as a major risk factor for a range of metabolic diseases and disorders including cardiovascular diease (Kenchaiah et al. 2002), diabetes (Resnick et al. 2000), and cancer (Bhaskaran et al 2014), and a substantial economic burden on health services (Withrow & Alter 2010; Wang et al. 2011).

An increasing number of studies have investigated the drivers of obesity, as well as possible dietary interventions to lessen the negative impacts associated with obesity (Kearney, 2010; Swinburn et al. 2011; Malik et al. 2013). From a nutritional perspective, it is acknowledged that obesity is a multifaceted issue, with a plethora of contributing dietary factors (Malik et al. 2013), but energy imbalance retains much of the main focus (Swinburn et al. 2011). On this topic, current evidence suggests that energy intake, rather than expenditure, is more important in driving obesity (Westerterp & Speakman 2008; Swinburn et al. 2009a; Swinburn et al. 2009b). This has led to a focus on caloric intake as a driver of obesity (Bleich et al. 2008; Shelley 2012), with recent debate on the relative contributions of fat and carbohydrate intake (Samaha et al. 2003; Austin et al. 2011; Te Morenga et al. 2013: MacGregor & Hashem 2014). Other studies have, however, argued that such single macronutrient approaches are overly simplistic and ignore the underlying complexity of the role of nutrition in obesity (Jebb & Prentice 2001; Simpson & Raubenheimer 2012).

One approach becoming increasingly prominent in obesity research is the Nutritional Geometric Framework (NGF) (Simpson & Rabenheimer 2012). The NGF is a state-space modelling approach that uses a range of diets in a multidimensional nutritional space to allow studies to examine the separate and combined effects of multiple macronutrients, rather than focussing on individual macronutrients (Simpson & Raubenheimer 1993). This framework has provided evidence that the balance and interactions of macronutrients are more important than the effects of individual macronutrients or total caloric intake in a number of important traits including

immunity (e.g. Cotter et al. 2011), lifespan (e.g. Maklakov et al. 2008), and reproduction (e.g. Lee et al. 2008) in a range of animal species. The NGF can also be used to investigate how individuals regulate their macronutrient intake, and what rules of compromise occur when individuals are constrained from consuming their preferred dietary ratio (Simpson *&* Raubenheimer 1999; Lee et al. 2002; Behmer, 2009). In a diversity of species, protein appears to be the most tightly regulated macronutrient (Raubenheimer & Simpson 1997; Raubenheimer et al. 2005; Simpson & Raubenheimer, 2005; Felton et al. 2009; Gosby et al. 2011, 2014). In light of changing human diets, where relative contributions of carbohydrates and fat have increased whilst protein has remained comparatively constant (Eaton 2006; Austin et al. 2011), this tight regulation of protein could have substantial implications for obesity and health by driving an overall increase in intake (Simpson & Raubenheimer 2005). Although dietary preferences and regulation can evolve (Warbrick-Smith et al. 2006), the rapid change in human nutritional environment has likely outpaced any response in metabolism and nutritional preferences, potentially leading to maladaptive dietary choice (Gosby et al. 2014). The prospect of separate regulatory networks for individual macronutrients (Berthoud & Seeley 2000) further illustrates the importance of a multiple macronutrient approach which considers both absolute and relative amounts of macronutrients contributing to the nutritional condition of individuals.

Until recently, the NGF has been fundamentally a two macronutrient approach, focussing usually on protein and carbohydrates (Lee et al. 2008; Maklakov et al. 2008; South et al. 2011 but see Jensen et al. 2012 that examines protein and lipid intake in an insect predator). This has partly restricted the application of this approach to obesity, as three macronutrients play a large dietary role in higher orders of species such as rodents and primates, compared to the two in most insect species the NGF has focussed on (Piper et al. 2011). Several extensions of the NGF have recently been developed to allow three macronutrients to be manipulated simultaneously (Solon-Biet et al. 2014; Chapter 1). These are already beginning to be applied to obesity related traits, with macronutrient balance being shown to be more important than total energy intake in the metabolic health and body fat of mice (Solon-Biet et al. 2014). With these improvements the NGF has great potential in

providing a robust framework to answer key questions in obesity involving the consequences of macronutrient balance and the role of dietary choice.

Here, I have used the extended NGF (Chapter 1) to investigate the consequences of protein (P), carbohydrate (C) and lipid (L) intake on lipid deposition in male and female cockroaches *Nauphoeta cinerea*. The approach allows simultaneous manipulation of all three macronutrients to analyse the independent effects of macronutrients, as well as the effects of macronutrient balance. Insects can be useful models to investigate obesogenic conditions and the consequences of changing proportions of macronutrients (Schilder & Marden, 2006; Warbrick-Smith et al. 2006; Trinh & Boulianne, 2013). Using the widely applicable NGF to study these effects provides an opportunity for consistency between studies and therefore better comparison between species. This study provides insight into how the balance of macronutrient intake influences lipid deposition, rather than just individual macronutrients or total energy intake.

3.3 METHODS

Animal collection

Male and female final instar nymphs were collected from large cultures of populations known to have high levels of genetic variation and no evidence of inbreeding (Corley et al. 2001). They were placed in single sex cultures with *ad lib* rat chow and water in large test tubes. Each day newly eclosed males and females were randomly allocated to be either focal individuals fed experimental diets, or part of mating assays. Non-focal individuals were stored in individual plastic boxes (11 x 11 x 3 cm) with *ad lib* rat chow food and water to be used only used once in mating assays. Focal individuals were housed in individual plastic containers (17 x 12 x 6 cm) with their diet and *ad lib* water. All animals were housed in a constant temperature room at 28°C with a 14L:10D lighting regime.

Design and production of experimental diets

A total of 300 artificial, holidic diets were produced, varying in percentage composition of P, C and L, as well as total nutritional content. Uncorrelated values for P, C and L were generated using a method outlined in Brooks et al. (2005). For

each macronutrient in each diet a random number was generated and converted to the inverse of a point on a normal distribution. This was then multiplied by a standard deviation of 75 and added to a mean of 50 to generate the final value. This mean and standard deviation was chosen to provide a wide spread of values encompassing the whole nutritional space. For each diet the sum of P, C and L was between 0-96.5% to allow a fixed composition of micronutrients at 3.5%. Any difference between the sum of the macronutrients and 96.5% was made up with cellulose, of which digestion is likely to be minimal in most species, including cockroaches (South et al. 2011). Britannia Finest Beef Dripping (100% animal fat with no added salt or antioxidants) was used as a pure source of lipid. A solid form of lipid was chosen to result in a solid consistency for all diets. Full details on the source of P, C, L and micronutrient mix of the diets is available in the supplementary material S1 of chapter 1. Distribution of the diets in the nutritional space is represented in Figure 3.2.

Dietary allocation and feeding

A single cockroach of each sex were allocated at random to each of the 300 diets on the day they eclosed to adulthood. Diets were dried in an oven at 30°C for 3 days to remove moisture and weighed on an electronic balance (Ohaus Explorer® Pro) to provide each cockroach was provided with approximately 250mg of diet. This amount was sufficient to ensure that cockroaches did not fully consume their diet during each feeding period. Diet was placed in a feeding platform that consists of an upturned vial lid (1.6cm diameter) glued on a petri dish (5.5cm diameter) so that any diet spilt during feeding could be collected. New diet and fresh water was provided every 5 days (a single feeding period) and the old diet was dried at 30°C for 3 days to remove water prior to re-weighing. Diet consumption was calculated for each feeding period by subtracting the final dry weight of diet after feeding from the original dry weight provided to each cockroach. The intake of P, C and L was then calculated from total intake using the percentage composition of the diet.

In total, males received a total of 11 feeds (i.e. 55 days post-eclosion) before being placed an Eppendorf and frozen at -80°C for lipid analysis (see below). Females received feedings until they gave birth to offspring or until they had received 11 feeds (in the case of females that aborted their clutch). Females were also placed

in individual Eppendorfs and frozen at -80°C for lipid analysis (see below). Nutrient intake was summed across the total number of feeds that each cockroach received. Due to our uneven temporal sampling regime across the sexes, nutrient intake was expressed per day of feeding and this measure was used in all subsequent statistical analyses.

Male and female mating protocol

After two feeding periods (i.e. 10 days post-eclosion), each experimental female was randomly allocated a 10 day old, virgin male as a mating partner. After diet had been removed, males were added to the empty plastic contained with the female and copulation monitored, with successful copulation being defined as a mating that lasted at least 10 minutes. In the event of successful copulation not occurring, a new male was used as a replacement and this process continued until a successful copulation resulted for all females. Mated males were returned to the large cultures, and females were placed back in their containers with fresh diet and water. Thirty one females aborted their clutch during the experiment, leaving a total sample size of n = 269 females with measures of nutrient intake and a clutch of offspring.

After two feeding periods (10 days-post eclosion), all focal males were randomly allocated a virgin, 10 day old, virgin female for measurement of male attractiveness (Chapter 1). The attractiveness of each male was measured in this way for a total of 4 times during the experiment (days 10, 20, 30 and 40 post-eclosion).

Lipid extraction

Males and females were defrosted and weighed to obtain a wet weight. A slit was then made in the abdomen to expose the fat body without piercing it, and pronotum width was measured using a dissecting microscope and eyepiece graticule. Females were then placed in glass vials and dried in the oven at 60°C for 48 hours and weighed again to obtain a dry weight. A 20ml solution of 2:1 dichloromethane:methanol mix was added to each vial to dissolve the lipids and hold them in solution. The vials were then placed in a shaker for two days before the liquid was drained and the individuals placed back in the drying oven at 60°C for a further two days. A final weight was recorded, and subtracted from the dry weight as a measurement of lipid reserves of the individual.

Statistical analysis

Full details on statistical analyses are provided in Chapter 1. In brief I used a multivariate response surface approach to estimate the linear and non-linear effects of the three macronutrients on lipid deposition (Lande and Arnold 1983) using a multiple regression model. From this a matrix of the quadratic and correlational terms of the macronutrients is derived. In order to avoid underestimation of these nonlinear effects (Phillips and Arnold 1989; Blows and Brooks 2003) canonical rotation was used to produce nutritional eigenvectors representing the major axes of non-linear effects. The magnitude and significance of the slope of these eigenvectors was calculated using double regression (Bisgaard and Ankenman 1996). The magnitude and significance of the curvature of these eigenvectors was calculated using a permutation procedure (Reynolds et al. 2009). Thin-plate splines (Green and Silverman 1994) were used to visualize the major eigenvectors as a nutritional landscape showing any peaks in lipid deposition. A sequential model building approach (Draper and John 1988) was used to determine if the linear and non-linear effects of nutrient intake on lipid deposition differed between the sexes and between females and their offspring.

3.4 RESULTS

The effect of macronutrient intake on lipid deposition

There were significant positive linear effects of C and L and a negative linear effect of P on female lipid deposition (Table 3.1A). There was also a positive quadratic effect of C and a significant positive correlational effect of the interaction between C and L (Table 3.1A). Canonical analysis of $\boldsymbol{\gamma}$ revealed three nutritional vectors with significant curvature ($\mathbf{m_1}$, $\mathbf{m_2}$ and $\mathbf{m_3}$) (Table 3.2A). The dominant nutritional vector ($\mathbf{m_3}$) was heavily loaded to the intake of C and L (both positive) and $\boldsymbol{\lambda}$ was negative indicating a peak in lipid deposition along this vector. There was also a significant positive linear slope ($\boldsymbol{\theta}$) on this vector favouring increases in C and L and a slight decrease in P (Table 3.1A). The second nutritional vector ($\mathbf{m_2}$) was also heavily loaded to C and L and $\boldsymbol{\lambda}$ was negative indicating a peak in lipid deposition along this vector. The third nutritional vector ($\mathbf{m_1}$) was heavily loaded to the intake of P

(negative) and to a much lower extend C (positive) and **λ** was positive indicating a trough in lipid deposition along this vector. There was also a significant negative linear slope (**θ**) on this vector favouring a large decrease in P and a slight decrease in C. Visualization of the nutritional landscape (Figure 3.1B) along the two main nutritional vectors ($\mathbf{m_2}$ and $\mathbf{m_3}$) shows a peak in female lipid deposition at positive values of $\mathbf{m_2}$ (high intake of L and low intake of C) and $\mathbf{m_3}$ (high intake of C and L).

There were significant positive linear effects of C and L and a negative linear effect of P on male lipid deposition (Table 3.1B). There were also significant positive quadratic effects of C and L as well as a positive correlational effect of the interaction between C and L (Table 3.1B). Canonical analysis of **γ** revealed three nutritional vectors with significant curvature ($\mathbf{m_1}$, $\mathbf{m_2}$ and $\mathbf{m_3}$) (Table 3.2B). The dominant nutritional vector ($\mathbf{m_3}$) was heavily loaded to the intake of C (negative) and L (positive) and **λ** was negative indicating a peak in lipid deposition along this vector. The second nutritional vector ($\mathbf{m_2}$) was heavily loaded to the intake of C and L (both positive) and **λ** was negative indicating a peak in lipid deposition along this vector. There was also a significant positive linear slope (**θ**) on this vector favouring increases in both C and L (Table 3.1B). The third nutritional vector ($\mathbf{m_1}$) was heavily loaded to the intake of P and **λ** was positive indicating a trough in lipid deposition along this vector. There was also a significant negative linear slope (**θ**) on this vector favouring a large decrease in P. Visualization of the nutritional landscape (Figure 3.1A) along the two main nutritional vectors ($\mathbf{m_2}$ and $\mathbf{m_3}$) shows a peak in male lipid deposition at positive values of $\mathbf{m_2}$ (high intake of both C and L) and values of $\mathbf{m_3}$ close to 0.

Comparison of the effects of macronutrient intake on lipid deposition across the sexes

There are linear, quadratic and correlational differences in the landscapes for male and female lipid deposition (Table 3.3). The linear differences are due to female lipid deposition being much more responsive to both P and L (Table 3.2). The quadratic differences are due to the curvature for both C and L being stronger for females than males. The correlational differences are due to an increase in both C and L having a positive effect on male lipid deposition but not female lipid deposition (Table 3.2).

3.5 DISCUSSION

Here, I investigated the consequences of macronutrient intake on lipid deposition in male and female cockroaches (*Naupheota cinerea*). My findings demonstrate that male and female lipid deposition is independently affected by the intake of all three macronutrients (P, C and L). P intake had a negative effect on lipid deposition, and both C and L intake had positive effects on lipid deposition, although there were clear differences between the sexes in the magnitude of these effects. In both sexes, lipid deposition was not maximised at a high intake of calories, a finding that challenges the established dogma on energy balance and obesity in humans (Swinburn et al. 2009) but is in general agreement with a recent study in mice (Solon-Biet et al. 2014). There is now substantial evidence that the balance of macronutrients is of high importance in lipid deposition and obesity in many species (Blumfield et al. 2012; Huang et al. 2013; Solon-Biet et al. 2014), in contrast to the single macronutrient or caloric views that still tend to dominate human health policy (Jebb et al. 2013).

For both sexes, the dominant nutritional vectors ($\mathbf{m}_2$ and $\mathbf{m}_3$) were heavily loaded to C and L intake, indicating the importance of these macronutrients to lipid deposition. This is not particularly surprising, as cockroaches store ingested C as L (Nation, 2001), but the finding that low P intake independently reduces lipid deposition is more unexpected. This finding could provide indirect support for the protein leverage hypothesis, whereby individuals that are able to reach their protein intake target without having to over-consume C and L deposit less fat. This is found in rats, where those on a high P:C ratio diet consume less total energy and consequently deposit less fat (Blouet et al. 2006). Alternatively, there may be direct effects of a high P diet that lead to low fat reserves, such as energetic costs of removing excess protein (Chen et al. 1999), and immunosuppression (Zheng et al. 2004). High P intake is known to decrease lifespan in cockroaches (Mullins & Cochran 1975) which may at least partly be explained by negative impacts on fat reserves. Steep costs of excess P intake on fat deposition may help explain the tight regulation of P often observed in many species including some fish (Raubenheimer et al. 2005), insects (Raubenheimer & Simpson 1997), spider monkeys (Felton et al 2009) and humans (Gosby et al. 2011, 2014).

Male and female cockroaches responded differently to macronutrient intake. Female lipid deposition was more responsive to L intake than males, and the curvature for both C and L was stronger, although only male lipid deposition increased with the covariance between C and L intake. Overall, female lipid deposition was more related to intake of lipids themselves, whreas in males the combined intake of C and L were important. There are likely to be various factors which contribute to this observed sex difference. The sexes likely have different nutritional needs due to differing strategies for maximising fitness, resulting in sexual divergence in the effects macronutrient intake (Hunt et al. 2004; Adler et al. 2013). In insects, females may allocate fewer carbohydrates to fat reserves than males due to the greater importance of energy reserves for male reproductive effort (Maklakov et al. 2008). Likewise, males may allocate a larger proportion of their lipid reserves to reproduction. Sexual selection in this species is largely driven by sex pheromone production (Moore & Moore 1999), of which lipids have a large effect (Chapter 1). There may also be a role of post-ingestive regulation, whereby absorption or utilization efficiency of nutrients can vary. Adaptive changes in nutrient assimilation can evolve in response to diet (Riha & Luckinbill 1996; Warbrick-Smith et al. 2006), thus differing selection on males and females could have resulted in divergent adaptive changes in post-ingestive regulation (Lee, 2010). Further work is needed, however, to test between these alternative explanations.

In Chapter 1, I showed that males and females regulate their intake to almost the same point in macronutrient space when given dietary choice, with a P:C:L ratio of 1:3.83:1.47 in females and 1:3.84:1.51 in males. Moreover I showed that this shared regulated intake point matches female reproductive fitness traits much better than it does male reproductive fitness traits. Here, I show that the regulated intake point closely matches the peak for female lipid reserves (Figure 3.1B) but not the peak for male lipid reserves (Figure 3.1A). Generally in insects there is a positive relationship between fat reserves and fitness through benefits to traits such as fecundity (Arrese & Soulages 2010), starvation resistance (Goenaga et al. 2013), flight duration (Beenakkers et al. 1984) and reproductive effort (Maklakov et al. 2008). There are, however, often trade-offs with traits such as lifespan and development time (Chippindale et al. 1996; Ballard et al. 2008; Barrett et al. 2009) and costs of extreme C intake (Raubenheimer et al. 2005). In contrast to the close match between

regulated intake point and lipid reserves in females, the regulated intake point in males occupies a region of low lipid deposition on the nutritional landscape (Figure 3.1A). Previous work showing that males chose a diet that maximises their lipid reserves (South et al. 2011) suggests that lipid reserves may be important for male fitness, through energetic displays and the production of sex pheromones, to which females are highly responsive (Moore et al. 1997). There are several possible explanations for the discrepancy in consequences of male dietary choice between this study and South et al. (2011). Firstly, here I have extended the manipulations to three macronutrients (P C & L), rather than the two (P & C) that South *et al* used. This may provide a more complete picture of nutrient regulation; revealing constraints on males, possibly due to shared genetics of choice with females, to choose a diet that doesn't maximise their lipid reserves (Maklakov et al. 2008). Alternatively, males may simply be behaving maladaptively, being unable to take advantage of lipids to maximise their lipid deposition in the same way they do when only carbohydrates and protein are available. Little is known about the nutritional ecology of *N. cinerea* in the wild, but they may be unlikely to ever encounter large, reliable sources of lipid to consumed, although they have been seen consuming the fat bodies of other cockroaches in colony (*pers. obs*.). Further work to gain a clearer picture of the fitness consequences of the regulated intake point will elucidate any constraints and conflicts that are occurring, as well as any trade-offs that occur between different traits that have divergent nutritional optima. For example, in *Drosophila melanogaster* females regulate their intake of P and C to a ratio that is intermediate to the divergent ratios that maximize lifespan and fecundity and this enables them to maximise lifetime reproduction (Lee et al. 2008).

In this study I have shown the importance of macronutrient balance for lipid deposition in male and female *N. cinerea*. Obesity is often thought of as a strictly human problem, with a combination of virtually limitless energy dense food and a lack of selection on the upper limits of body fat due to predation risk a possible genetic path to obesity (Speakman 2007, 2008). Obesity does occur, however, in many other animal species. For example, bears consume high L diets and gain substantial weight before hibernation (Erlenbach et al. 2014) without the accompanying increased insulin sensitivity usually found in humans (Nelson et al. 2014). Many species of migratory birds are butterflies also become heavily obese,

with as much as 50% of their body mass made of fat before the prolonged endurance exercise of migration (McWilliams et al. 2004; Satterfield et al. 2013). Understanding the physiology of how some species deal with high L intake and weight gain without any apparant negative consequences can provide insight and novel treatments for humans. Several species of rodents and insects have also been used to model obesity, looking at the effects of macronutrients, genetic factors and nutrient regulation (Lutz & Woods 2012; Nilsson et al. 2012). Complete modelling of obesity requires understanding the links between nutrient regulation and the effects of macronutrients, and the underlying genetic factors. *Drosophila* can be a fruitful model given their analogous metabolic and regulatory systems to humans, and ability to store and utilize substantial fat reserves (Baker & Thummel, 2007; Liu et al. 2012). The extended NGF provides an integrated, nutritionally relevant framework to examine the effects of macronutrients in these models as well as other species, and test the universality of effects.

Defining and designing balanced diets is a central tenet in nutritional ecology research but is an extremely complex task. Obesity has a substantial genetic component (O'Rahilly & Farooqi, 2006) involving many satiety and appetite genes (O'Rahilly & Farooqi, 2006; Speliotes et al. 2010) and significant genotype by environment (GxE) interactions (Garver 2011; Kilpeläinen et al. 2011). Genes involved in appetite control or nutrient signalling pathways, such as TOR, are prime candidates to be involved in GxE interactions, as these pathways are responsive to nutrient levels and balance (Ribeiro & Dickson, 2010; Vargas et al. 2010). The NGF, with its appreciation for the independent and combined effects of macronutrients, provides the potential to gain further insight into the overall importance of these factors in obesity. Traits such as nutrient signalling activation can be mapped against nutritional landscapes to understand the role these factors play in individual's varied responses to macronutrients. A greater understanding of the way responses to macronutrients differ according to factors such as genetics, sex and GxE interactions could lead to advances in nutrition and the concept of balanced diets through personalization of dietary advice (Qi 2014). Future work applying the NGF can also provide important insight into the way levels of macronutrients are regulated. Many species regulate macronutrients separately (Raubenheimer & Simpson, 1997; Simpson & Raubenheimer, 2000; Sorensen et al. 2008), and understanding how

these regulatory systems interact in a diet can shed light on factors driving obesity. The protein leverage hypothesis offers a testable hypothesis for nutrient regulation (Gosby et al. 2014), and understanding such a process has great potential in combatting obesity, which seems to be largely driven by food intake (Swinburn et al. 2009a; 2009b).

Nutrition plays a large role in health in factors such as ageing and lifespan (Lee et al. 2008; Nagakawa et al. 2012) and obesity and metabolic disease (Swinburn et al. 2009b; Huang et al. 2013). It is becoming increasingly apparent that macronutrient balance fundamentally underpins many of these aspects of health (Simpson & Raubenheimer, 2012; Solon-Biet et al. 2014). Studies on insects and rodents have shown a role of nutrient signalling pathways such as TOR and IGF in mediating these responses (Simpson & Raubenheimer 2009; Oldham 2011; Solon-Biet et al. 2014). Improvements to the NGF allow this approach to play a major role in understanding the full role of macronutrient balance in the mosaic of traits that define health, and establishing whether relationships observed in insects and rodents hold true in humans.

FIGURE LEGENDS

Figure 3.1. Graphic representation of the two major axes of non-linear effects from canonical analysis on male (A) and female (B) lipid deposition. Each point represents one cockroach. The regulated intake point of females is shown in white.

Figure 3.2. The distribution of artificial, holidic diets in nutritional space. Individual diets (grey symbols) are mapped in (A) protein versus carbohydrate, (B) protein versus lipid and (C) carbohydrate versus lipid nutritional space.

Figure 3.1

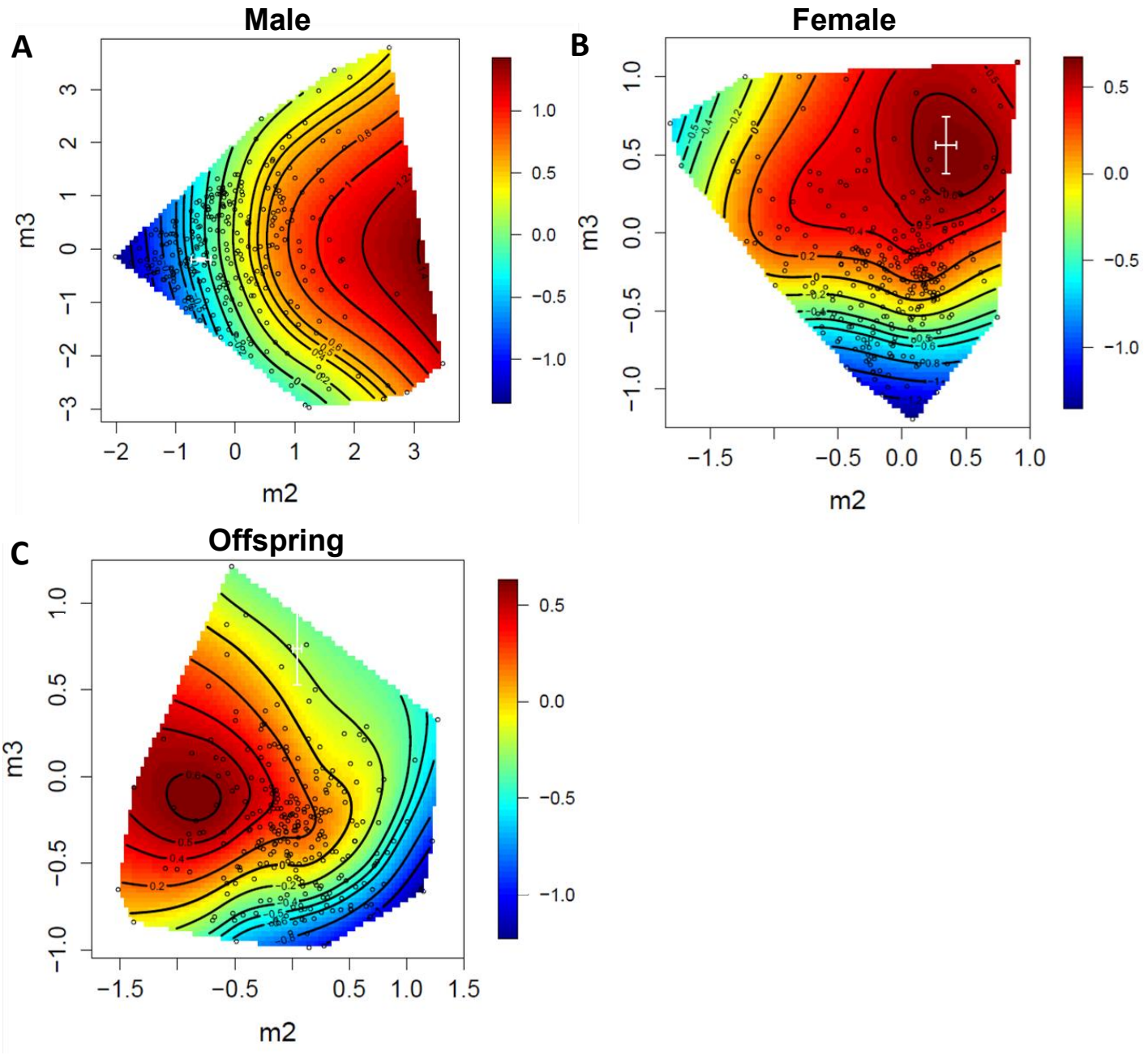

Figure 3.2

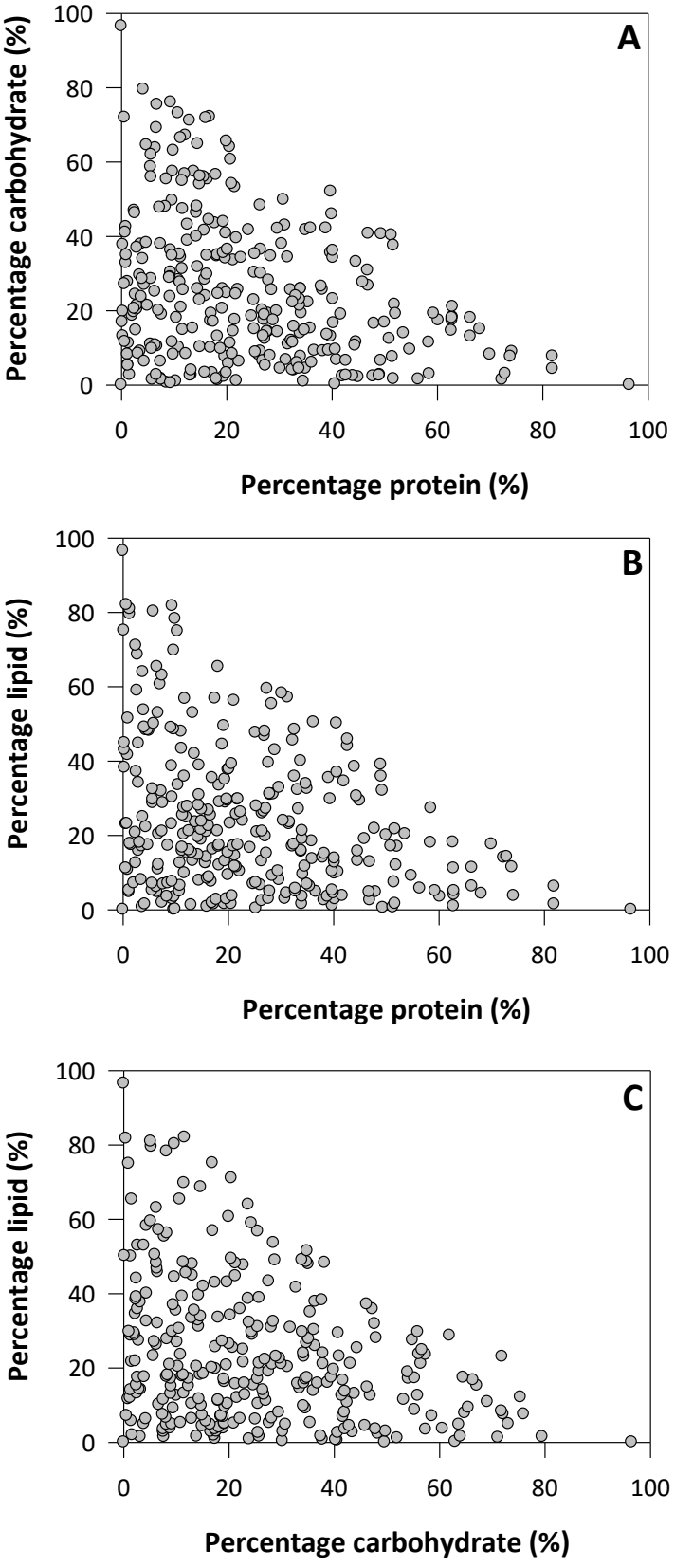
A
Percentage carbohydrate (%)
Percentage protein (%)
B
Percentage lipid (%)
Percentage protein (%)
C
Percentage lipid (%)
Percentage carbohydrate (%)
0
20
40
60
80
100

TABLE LEGENDS

Table 3.1. The linear and nonlinear coefficients (±SE) of protein (P), carbohydrate (C) and lipid (L) intake on lipid deposition in (A) female (B) male and (C) Offspring *N. cinerea*. In **γ**, quadratic terms are given along the diagonal (P x P, C x C and L x L) and correlational terms (P x C, P x L and C x L) in the off-diagonal position. Significance test: * $P < 0.05$, ** $P < 0.001$, ** $P < 0.0001$.

Table 3.2. The **M** matrix of eigenvectors ($\mathbf{m_1}$ to $\mathbf{m_3}$) from the canonical analysis of **γ** for lipid deposition in (A) female and (B) male *N. cinerea*. The linear ($\boldsymbol{\theta}_i$) and quadratic ($\boldsymbol{\lambda}_i$) terms describe the slope and curvature of a given nutritional vector, respectively, and are provided in the last two columns. Significance test: * $P < 0.05$, ** $P < 0.001$, ** $P < 0.0001$.

Table 3.3. Sequential model building approach comparing the linear (P, C, L), quadratic (P x P, C x C, L x L) and correlational (P x C, P x L, C x L) effects of nutrient intake on lipid reposition between the sexes in *N. cinerea*. Whenever an overall effect is significant, univariate tests are provided to determine which nutrient(s) contributes to this effect (denoted by letter in superscript, statistics provided in table footer).

Table 3.1

		Y		
Nutrient intake	Linear Effects	P	C	L
H. Females				
P	-0.64 ± 0.13***	0.20 ± 0.22		
C	0.26 ± 0.12*	0.54 ± 0.30	-0.53 ± 0.19*	
L	0.70 ± 0.14***	-0.09 ± 0.38	-0.57 ± 0.31*	-0.59 ± 0.22
I. Males				
P	-0.31 ± 0.05***	0.06 ± 0.03		
C	0.25 ± 0.05***	0.04 ± 0.07	-0.09 ± 0.03*	
L	0.33 ± 0.05***	-0.01 ± 0.05	0.17 ± 0.06*	-0.11 ± 0.03**

Table 3.2

	M				Nutritional effects	
	P	C	L		θ_i	λ_i
H. Females						
m_1	0.92	0.35	-0.16		-0.61***	0.62***
m_2	0.36	-0.62	0.70		0.10	-0.71***
m_3	-0.15	0.70	0.70		0.76***	-1.74***
I. Males						
m_1	0.99	-0.11	0.02		-0.27***	0.13 **
m_2	-0.11	0.74	0.67		0.39***	-0.04 ***
m_3	0.07	-0.67	0.74		0.10	-0.38***

Table 3.3

	SS_R	SS_C	DF_1	DF_2	F	P
A. Male versus Female Lipid Deposition						
Linear	424.56	410.27	3	554	6.43	0.0003[A]
Quadratic	379.52	372.01	3	548	3.69	0.01[B]
Correlational	368.10	361.09	3	542	3.51	0.01[C]

Univariate tests: [A] P: $F_{1,554} = 5.81$, $P = 0.02$; C: $F_{1,554} = 0.003$, $P = 0.96$; L: $F_{1,554} = 6.94$, $P = 0.009$; [B] PxP: $F_{1,548} = 1.81$, $P = 0.18$; CxC: $F_{1,548} = 4.97$, $P = 0.03$; LxL: $F_{1,548} = 5.03$, $P = 0.03$; [C] PxC: $F_{1,542} = 2.89$, $P = 0.09$; PxL: $F_{1,542} = 0.04$, $P = 0.85$; CxL: $F_{1,542} = 6.10$, $P = 0.01$.

4. GENERAL DISCUSSION

The large impact of nutrition on fitness-related traits is often caused by complex interactions and/or a balance between the intake of macronutrients, and therefore are not representable by single axis of nutrition: be that calories or a single macronutrient (Simpson & Raubenheimer 2012). A large collection of studies have used the Nutritional Geometric Framework (NGF) to demonstrate the role of macronutrient balance in a range of traits such as immunity (Cotter et al. 2011; Ponton et al. 2013), predator foraging choices (Mayntz et al. 2009; Jensen et al., 2012) and reproduction and lifespan (Lee et al., 2008; Simpson & Raubenheimer, 2009). The NGF has undoubtedly had a huge impact on the way nutrition is studied, providing new evidence to aid in resolving longstanding evolutionary debates (Lee et al., 2008; Mayntz et al. 2009; Solon-Biet et al. 2014). Many advances have been made, and in recent years the advent of mixture triangles have furthered the reach of the NGF in field studies and optimizing commercial animal feeds (Johnson et al. 2013; Cowieson et al. 2014; Raubenheimer et al. 2015). These studies have shown promise and demonstrated that macronutrient balance is a universal way in which nutrition should be studied, and that even simple nutritional situations can be influenced by multiple macronutrients (Simpson & Raubenheimer 2005; Gosby et al. 2011, 2014).

Despite this great progress there are key limitations of the NGF which are likely to hinder further progress. There is a clear need for a multidimensional approach that can incorporate with the nutritional environment experienced by more complex organisms, particularly those used as models for human health and disease. The NGF in its current form is unable to meet this demand, and although existing mixture triangles have been a highly useful development in many areas, they also have restricted ability to answer many emerging questions in the importance of nutrition. My research aimed to counter these limitations and restrictions by developing an extension to the NGF. In this discussion I will summarize the results of my tests of this new framework, and place these results, and the new framework as a whole, in the context of both current and potentially fruitful future research.

4.1. EXTENDING THE NUTRITIONAL GEOMETRIC FRAMEWORK

Nutritional research has long since moved past an approach using a single currency, such as energy intake, to model the effects on important fitness-related traits. The NGF has been integral in this philosophy change, providing a simple and transferrable framework for studies to manipulate two macronutrients and investigate the independent and interactive effects of those macronutrients (Simpson & Raubenheimer 1993). The growing numbers of studies using this approach have consistently demonstrated the importance of macronutrient balance in a range of key areas (Lee et al. 2008; Ponton et al. 2013; Solon-Biet et al. 2014). Key limitations in the design of diets and restricted dimensionality have, however, limited the application of the NGF to several areas where it can be of greatest use. Alternative developments of the framework have important uses, but lack the universal applications of the original framework. In Chapter 1, I presented an extension to the NGF that aimed to solve these key limitations, while keeping the main advantages of this approach. I aimed to argue the broad applications of the new framework to emerging and longstanding questions in nutrition, whilst also demonstrating its use on a study of reproduction in male and female cockroaches (*Nauphoeta cinerea*).

A key finding of this chapter was that male sex pheromone production was maximised at a different macronutrient balance to pre-copulatory attractiveness, which required high levels of both lipid and carbohydrate, compared to lipids which are mainly influenced by lipid intake. Previous work using two macronutrients has found the two traits to be maximised at the same nutrient balance (South et al. 2011), and using three macronutrients has provided extra insight into these traits, which may reflect an increased energetic component of pre-copulatory attractiveness that requires a different nutritional intake than sex pheromones (Moore & Breed 1986). This demonstrates the separate importance of carbohydrates and lipids, which are sometimes thought of interchangeable as rapid sources of energy (Raubenheimer 2009), validating the use of a three-dimensional framework even in relatively simple nutritional situations such as this.

Recent work on this species using the NGF has suggested a possible trade-off between maximising pre- and post-copulatory success (Bunning et al. 2015) with carbohydrates of paramount important to pre-copulatory success, and protein of

higher relative importance to post-copulatory success due to the role of sperm number in male fertility (Bunning et al. 2015). This finding is corroborated here, and extra insight is added by showing how lipids might influence this trade-off. Lipids had marginal effects on post-copulatory success, which is still maximised at a high protein intake when considering all three macronutrients. There were however more profound effects of lipid on pre-copulatory success, which is partly determined by sex pheromone profiles. Both carbohydrates and lipids had profound effects on sex pheromone maximisation, but attractiveness was maximised at a higher carbohydrate:lipid ratio than sex pheromones alone. This may reflect an energetic component to courtship (Moore & Breed 1986) requiring more carbohydrates than would maximise only sex pheromones. The result of this may be a trade-off in maximising different components of pre-copulatory success, energetic courtship and sex pheromone profiles, mediated by lipid and carbohydrate intake. This highlights that carbohydrates and lipids should not be combined into one unit of nutrition, as they have diverse effects. Carbohydrates had higher impact on male fertility than lipids, but consuming more carbohydrates to alleviate the conflict between pre- and post-copulatory success may be constrained by the importance of lipid:carbohydrate balance in pre-copulatory traits such as courtship and sex pheromone expression. The need to distinguish between protein and carbohydrates is further illustrated by the finding of sex-specific effects of lipid:carbohydrate balance on fitness traits demonstrating that males and females respond differently to the balance of these two macronutrients. The extended NGF is of high value in adding resolution to these effects, which can aid in understanding underlying mechanisms. The nutritional choices faced by species are clearly complex, with conflicting demands needing to balance across multiple macronutrients.

Alongside the ability to improve studies of nutrition in insects and other taxa with relatively simple nutrition (Piper et al. 2011), the extended framework has large potential in numerous fields. A main reason for this is simply extending the range of study organisms that can be realistically modelled based on the nutritional constraints of the framework. This can be crucial in areas such as the longstanding question of whether dietary restriction is a universal lifespan extender, that could potentially be applied for use in human health. Using this extended framework will firstly allow dietary restriction to be tested in a nutritionally relevant way in a larger

range of organisms to determine if the effect is truly universal (Nagakawa et al. 2012). It can also provide additional evidence on which aspect of diet has to be restricted, calories or some other unit. Nutritional geometry in two dimensions has provided compelling evidence for protein to be the key element, rather than calorie intake in *Drosophila* (Lee et al. 2008; Jensen et al. 2015) and in three dimensions in mice, using mixture triangles (Solon-Biet et al. 2014) whereas experiments on calorie restriction in primates have provided mixed results (Mattison et al. 2012; Colman et al. 2014). Both the universality of the effect and the key unit of nutrition can be more accurately investigated with the extended framework, with the added benefit of being able to map data on potential underlying mechanisms such as TOR (Partridge et al. 2011) onto nutritional landscape. It is also theoretically possible to move to another level of nutrition, focussing on the constituent parts of protein, amino acids. Some of these have already been linked to dietary restriction and lifespan in elegant add-back experiments (Grandison et al. 2009) but it can now be possible to manipulate many amino acids simultaneously and separate the independent and correlational effects. The extended framework can also be a useful tool to complement mixture triangles in bringing nutritional geometry out of the lab and into the field, where there may be different pressures on nutritional decisions and more temporal and spatial variation in availability of different macronutrients. Mixture triangles have been highly useful in field studies as they rely on relative proportions of macronutrients consumed rather than absolute amounts (Johnson et al. 2013; Raubenheimer et al. 2015). The extended framework can complement this by examining the role of total nutritional level that is missed by mixture triangles, and linking intake data to key traits.

4.2. THE ROLE OF MACRONUTRIENT BALANCE IN LIPID DEPOSITION AND OBESITY

Obesity is an increasing global problem (Finucane et al. 2011; Ng et al. 2014), with profound implications on the economic costs of healthcare (Withrow & Alter 2010; Wang et al. 2011). Although there are many factors that influence any individuals propensity towards obesity, from genetics and genotype by environment interactions to exercise regime (O'Rahilly & Farooqi 2006; Speliotes et al. 2010; Garver 2011; Kilpeläinen et al. 2011), diet plays a significant role, and is one which can be easily

altered (Westerterp & Speakman 2008; Swinburn et al. 2009a; Swinburn et al. 2009b). Although it is often acknowledged that obesity is a multi-macronutrient problem (Malik et al. 2013), many studies still focus on energy imbalance or single macronutrients (Samaha et al. 2003; Austin et al. 2011; Swinburn et al. 2011; Te Morenga et al. 2013; MacGregor & Hashem 2014). The NGF has already provided some insight into this field, showing for example that macronutrient balance is more important in the fat deposition of mice than the total intake of nutrients (Solon-Biet et al. 2014). The protein leverage hypothesis is another way that nutritional geometry has contributed to obesity research (Simpson & Raubenheimer 2005; Gosby et al. 2011, 2014). There is now a wide range of species where protein is known to be the macronutrient that individuals will prioritize the regulation of, including primates (Felton et al. 2009) fish (Raubenheimer et al. 2005) and some insects (Raubenheimer & Simpson 1997). Whilst this is understandably difficult to study in humans, there is evidence that protein leverage occurs (Gosby et al. 2011, 2014), highlighting a potential large role for protein in explaining obesity. Protein has been largely neglected in obesity in favour of carbohydrates and lipids, which are more directly linked to obesity, but if protein leverage does occur, changing macronutrient ratios in human diets could explain patterns of overeating. Nutritional geometry can be used under controlled experimental conditions in the lab to reveal nutritional effects such as protein leverage (Simpson & Raubenheimer 1997) that may have widespread relevance across taxa. Whilst this hypothesis is yet to be widely tested, it represents another area where studies of macronutrient balance can add understanding outside of the study species used.

In Chapter 2 of this thesis, I used the extended framework I developed in Chapter 1 to investigate the independent effects of protein, carbohydrate and lipid intake, as well as interactions between these macronutrients, on lipid deposition in male and female cockroaches (*N. cinerea*). My aim was to show the potential of the extended framework to obesity research in explaining the nutritional basis of fat deposition, and show how this could be furthered to aid investigation of underlying mechanisms of obesity. I found that all macronutrients have significant independent effects on fat deposition in male and female cockroaches. Furthermore fat deposition was not maximised at the highest intake of energy; a finding that parallels a recent study in mice that demonstrated the importance of macronutrient balance (Solon-Biet et al.

2014). Protein is often predicted to have effects due to protein leverage, but here I found that fat deposition decreased with protein intake. These could be due to energetic costs of removing excess protein. Studies on livestock have shown growth and body fat to be slowed on high protein diets, potentially due to increased metabolic activity needed to remove nitrogenous waste (Chen et al. 1999; Rehfeldt et al. 2011). An alternative explanation comes from potential immunosuppression caused by high protein intake. In humans at least high protein intake can lower immunity through both hyper-stimulation and increased unresponsiveness of the immune system (Moreau & Coste 1993; Zheng et al. 2004). This could then lead to loss of appetite, as found in immune-challenged *Drosophila* (Ayres & Schneider 2009), possibly as an adaptation to boost survival chances in the face of immune challenges.

I found that the macronutrient balance that maximised fat deposition differed between males and females, with male deposition maximised at both high carbohydrate and high lipid intake, compared to females where fat deposition was maximised at high lipid intake. This may reflect different nutritional needs of males and females, that often show very different way of maximising reproduction (Maklakov et al. 2008; Morehouse et al. 2010; Reddiex et al. 2013). This is particularly interest given my finding that males and females share a regulated intake point. This is unexpected as males and females have clear differences in nutrient balances which maximise key fitness traits. Sexual conflict over intake may be occurring in *N. cinerea*, as has been found in black field crickets (Maklakov et al. 2008), with sexual divergence limited by the fact that males and females share a genome. There are, however, alternative ways males and females can differ in regulation, through post-ingestive regulation of nutrient assimilation and use efficiency (Raubenheimer & Simpson 2003; Clissold et al. 2010; Jensen et al. 2013) which warrant further study.

In male cockroaches, the regulated intake point did not match any of the fitness-related traits we measured, whereas for females this point matches the optima for fat deposition. Whilst in many species this leads to high fecundity (Arrese & Soulages 2010) this was not found to be the case here, although fecundity was only measured for the first clutch of offspring and not across the entire lifespan of the female (which can live for over 3 years). The starvation resistance and fecundity benefits of fat

deposition (Chippindale et al. 1996; Goenaga et al. 2013) may provide an adaptive benefit to females of choosing to maximise fat reserves. Alternatively, females may be behaving maladaptively, constrained by shared genetics and selection on the regulated intake of nutrients by males (Maklakov et al. 2008) or unable to choose efficiently in the presence of lipids, which may be an uncommon source of macronutrients in the wild. High fat deposition is usually thought of as costly in humans, and the benefits to many insect species (Arrese & Soulages 2010) may make these species a questionable model for human obesity, although high fat deposition can also be costly in insects (Warbrick-Smith et al. 2006).

The extended framework is applicable for use across many taxa, including those commonly used as models for human health such as rodents and primates (Nilsson et al. 2012; Lutz & Woods 2012; Colman et al. 2014). A multidimensional approach to obesity, alongside consistency in methodology and the ability to integrate macronutrient intake with candidate mechanisms make the extended framework of great potential value.

4.3. CONCLUSION

Importantly, the extension of the nutritional framework I outline in my thesis shares many of the aspects of the NGF which have made it so useful. Measured phenotypic traits can still be easily mapped onto nutrient intake to investigate the extent of any associations. This will be especially important in maintaining nutritional geometry as an integrative approach that aids in the bringing together of knowledge from different areas. Large volumes of research have been published on potential mechanisms underpinning many of the effects where nutrition has a large role, such as nutrient signalling pathways in both obesity (Ribeiro & Dickson 2010; Vargas et al. 2010) and the effects of dietary restriction on lifespan and reproduction (Kapahi et al. 2010 ;Partridge et al. 2011). The new framework has broad applications, but of particular importance is the potential to integrate studies on these mechanisms with studies performing dietary manipulations which could play a vital role in fully understanding potential interventions to human health (Simpson & Raubenheimer 2009). By understanding both the ideal interventions and the mechanisms underlying them, the potential exists for a healthy lifespan to be extended in humans. These benefits,

alongside an improved understanding of the fundamental link between nutritional intake and fitness are likely to provide widespread and profound advantages of this extended nutritional geometric framework.

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Appendix 1: Supplementary material S1

Text S1: Diet Ingredients

Artificial diets were created that varied in protein, carbohydrate and lipid content as well as total energetic content. The protein sources used were a 3:1:1 ratio of casein, albumen and peptone. Carbohydrate sources were a 1:1 ratio of sucrose and dextrin. The source of protein was Britannia Finest Beef Dripping, which consists of 100% animal fat (57g of unsaturated fat, 36g of mono-unsaturated fat and 3g of polyunsaturated fat per 100g). A fixed proportion (3.5%) of micronutrients were added to the pure sources of macronutrient to promote normal growth and function. This consisted of Wesson salt mix (2.5%), cholesterol (0.55%), vitamin C (0.28%), linoleic acid (<0.01%) and a vitamin mix (0.18%). The vitamin mix itself consisted of thiamine, riboflavin, nicotinic acid, pyridoxine, folic acid, meso-inositol, calcium pantothenate, p-aminobenzoic acid. Choline chloride and biotin. Any difference between the macronutrient limit of 96.5% and the sum of the percentage composition of protein, carbohydrates and lipids was made up with cellulose in crystalline form. Digestion of this form of cellulose and subsequent use as carbohydrate intake is highly likely to be minimal, and of now significant consequence (Jones and Raubenheimer 2001; Raubenheimer & Jones 2006).

Supplemental Figures

Figure S1. The distribution of artificial, holidic diets in nutritional space. Individual diets (grey symbols) are mapped in (A) protein versus carbohydrate, (B) protein versus lipid and (C) carbohydrate versus lipid nutritional space.

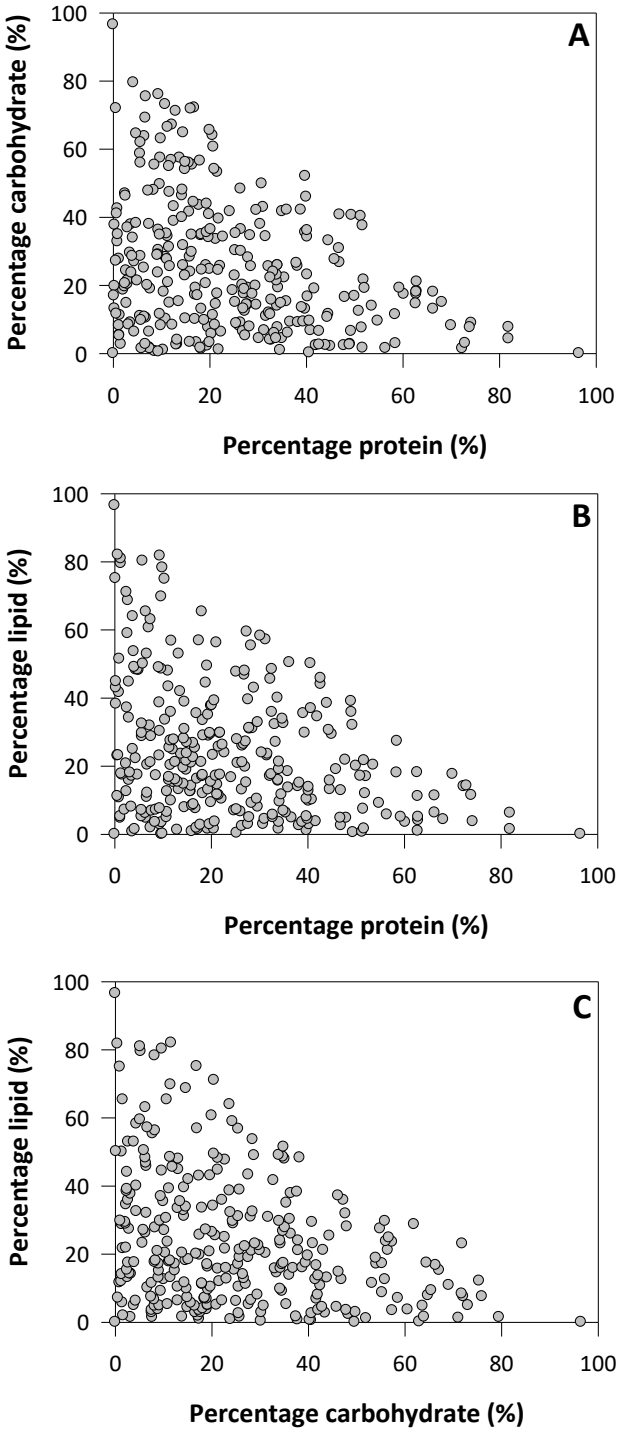
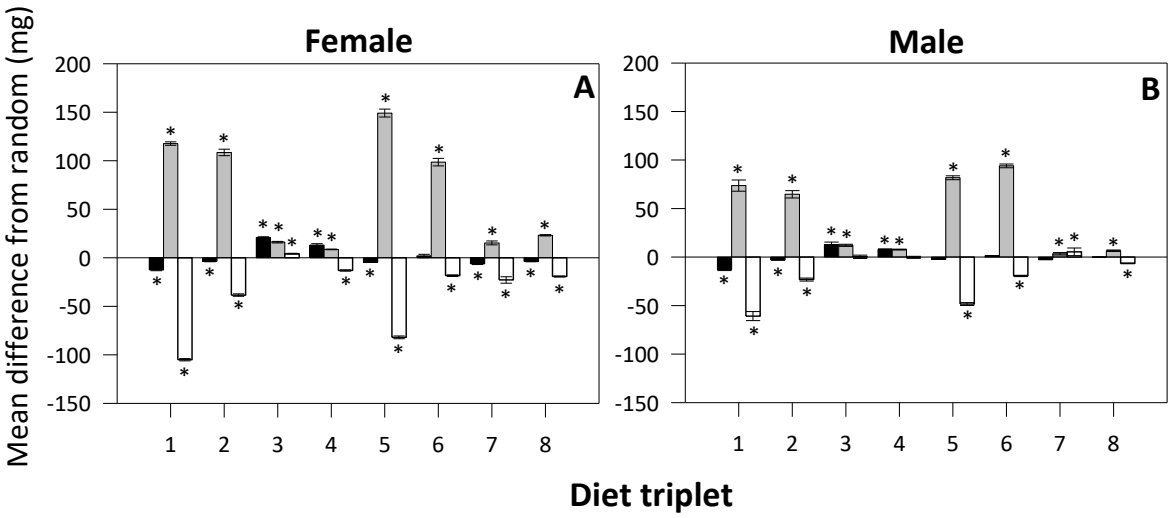


Figure S2. Mean (±SE) difference in the intake of protein (black bars), carbohydrates (grey bars) and lipids (white bars) from a random consumption of nutrients in each diet triplet for (A) female and (B) male *N. cinerea*. Asterisks indicate that the difference in nutrient intake is significantly different from zero (using a one-sample *t*-test).



Supplemental Tables

Table S1. The nutrient composition of the 300 artificial, holidic diets used in Experiment 1. %P, %C and %L represent the percentage of protein, carbohydrate and lipid in the diet, respectively. %TN is the percentage total nutrition of the diet, which reflects the sum of %P, %C and %L.

Diet	%P	%C	%L	%TN	Diet	%P	%C	%L	%TN	Diet	%P	%C	%L	%TN
1	0.0	96.5	0.0	96.5	**101**	6.8	75.4	12.1	94.3	**201**	5.7	61.9	28.7	96.3
2	96.5	0.0	0.0	96.5	**102**	15.8	41.5	12.5	69.8	**202**	7.6	1.4	28.7	37.7
3	9.7	49.6	0.0	59.3	**103**	15.7	55.9	12.5	84.1	**203**	19.5	2.2	28.8	50.5
4	9.9	63.0	0.1	73.0	**104**	10.9	10.1	12.5	33.5	**204**	18.4	24.5	28.9	71.8
5	25.3	30.2	0.3	55.8	**105**	2.5	46.9	12.5	61.9	**205**	19.9	40.8	29.3	90.0
6	49.4	40.6	0.5	90.5	**106**	40.2	23.1	12.7	76.0	**206**	45.0	2.1	29.3	76.4
7	51.3	40.3	0.6	92.2	**107**	46.9	26.7	12.8	86.4	**207**	5.7	55.9	29.6	91.2
8	3.7	37.8	0.7	42.2	**108**	26.4	9.0	13.0	48.4	**208**	21.8	24.4	29.6	75.8
9	16.0	23.9	0.8	40.7	**109**	19.4	43.9	13.0	76.3	**209**	19.8	34.0	29.6	83.4
10	62.8	17.4	0.9	81.1	**110**	13.3	2.6	13.1	29.0	**210**	39.4	9.3	29.7	78.4
11	39.8	52.0	1.1	92.9	**111**	44.6	11.5	13.1	69.2	**211**	21.9	1.1	29.7	52.7
12	13.0	71.1	1.2	85.3	**112**	36.0	42.1	13.6	91.7	**212**	9.4	36.2	30.2	75.8
13	17.3	3.2	1.4	21.9	**113**	40.2	36.1	13.8	90.1	**213**	44.4	10.6	30.5	85.5
14	4.2	79.5	1.4	85.1	**114**	72.3	1.4	14.0	87.7	**214**	14.5	31.7	30.8	77.0
15	81.9	7.7	1.4	91.0	**115**	38.0	26.5	14.0	78.5	**215**	28.0	28.1	30.9	87.0

16	34.0	13.9	1.5	49.4	**116**	15.9	3.3	14.1	33.3	**216**	28.6	25.5	31.0	85.1
17	19.3	25.8	1.5	46.6	**117**	72.9	3.0	14.2	90.1	**217**	27.9	14.3	31.1	73.3
18	20.6	64.0	1.5	86.1	**118**	21.3	33.5	14.5	69.3	**218**	7.3	47.7	31.8	86.8
19	51.7	37.5	1.6	90.8	**119**	14.3	46.3	14.7	75.3	**219**	49.3	6.5	32.0	87.8
20	17.0	17.2	1.8	36.0	**120**	38.3	9.1	15.0	62.4	**220**	33.2	23.9	32.2	89.3
21	7.5	1.7	1.9	11.1	**121**	27.3	12.4	15.1	54.8	**221**	5.7	28.5	32.4	66.6
22	26.4	48.3	2.3	77.0	**122**	12.2	67.1	15.2	94.5	**222**	35.0	4.4	32.5	71.9
23	0.0	0.0	96.5	96.5	**123**	20.0	24.7	15.3	60.0	**223**	29.7	14.2	32.8	76.7
24	38.2	25.6	2.5	66.3	**124**	13.1	2.5	15.3	30.9	**224**	18.3	13.0	33.4	64.7
25	46.9	40.7	2.5	90.1	**125**	33.9	21.3	15.6	70.8	**225**	10.5	18.0	33.5	62.0
26	17.8	43.5	2.7	64.0	**126**	44.6	33.1	15.7	93.4	**226**	34.8	14.7	33.8	83.3
27	40.3	16.6	2.8	59.7	**127**	11.3	34.3	15.8	61.4	**227**	3.0	20.3	34.1	57.4
28	19.7	7.6	2.8	30.1	**128**	33.9	23.0	15.8	72.7	**228**	42.0	2.3	34.5	78.8
29	9.4	30.3	2.8	42.5	**129**	3.0	36.9	15.8	55.7	**229**	19.4	35.5	35.0	89.9
30	27.7	17.7	2.9	48.3	**130**	12.6	38.8	16.0	67.4	**230**	17.0	10.0	35.4	62.4
31	30.8	49.8	2.9	83.5	**131**	17.1	19.2	16.2	52.5	**231**	39.1	13.5	35.4	88.0
32	9.8	57.4	3.4	70.6	**132**	24.2	41.7	16.6	82.5	**232**	32.6	22.2	35.8	90.6
33	38.9	42.1	3.5	84.5	**133**	11.3	66.4	16.7	94.4	**233**	11.7	47.3	35.8	94.8
34	8.4	47.9	3.5	59.8	**134**	18.2	34.6	16.9	69.7	**234**	49.1	2.7	35.8	87.6

35	60.2	17.4	3.5	81.1	**135**	52.1	19.1	16.9	88.1	**235**	40.7	9.5	36.9	87.1
36	34.1	7.6	3.5	45.2	**136**	32.2	25.5	17.0	74.7	**236**	2.6	46.2	37.1	85.9
37	20.8	60.6	3.6	85.0	**137**	50.9	12.4	17.1	80.4	**237**	20.0	3.2	37.6	60.8
38	41.7	19.0	3.7	64.4	**138**	21.0	54.1	17.1	92.2	**238**	20.2	36.4	37.8	94.4
39	74.2	8.9	3.7	86.8	**139**	3.6	9.0	17.1	29.7	**239**	0.3	37.7	38.2	76.2
40	62.9	18.2	4.0	85.1	**140**	8.6	55.3	17.2	81.1	**240**	44.0	2.4	38.4	84.8
41	68.1	15.0	4.3	87.4	**141**	18.3	34.9	17.3	70.5	**241**	9.3	23.7	38.6	71.6
42	31.1	42.9	4.4	78.4	**142**	1.6	2.7	17.4	21.7	**242**	14.5	25.8	38.8	79.1
43	40.0	45.9	4.4	90.3	**143**	4.8	64.5	17.4	86.7	**243**	49.0	2.5	39.0	90.5
44	48.0	16.5	4.7	69.2	**144**	21.9	39.5	17.4	78.8	**244**	20.7	11.2	39.2	71.1
45	46.8	30.8	4.7	82.3	**145**	32.5	4.0	17.5	54.0	**245**	27.7	14.3	39.5	81.5
46	1.2	8.2	4.7	14.1	**146**	70.0	8.2	17.6	95.8	**246**	33.8	4.4	40.0	78.2
47	6.5	63.7	4.7	74.9	**147**	1.4	11.2	17.7	30.3	**247**	0.9	32.8	41.6	75.3
48	36.7	9.2	4.8	50.7	**148**	58.4	11.4	18.0	87.8	**248**	13.6	15.2	41.9	70.7
49	32.8	3.9	4.9	41.6	**149**	3.2	8.4	18.0	29.6	**249**	28.9	17.4	42.9	89.2
50	10.8	73.1	4.9	88.8	**150**	62.7	14.6	18.1	95.4	**250**	0.3	19.7	43.0	63.0
51	27.9	19.2	4.9	52.0	**151**	35.9	15.2	18.4	69.5	**251**	11.2	27.6	43.3	82.1
52	59.3	19.2	5.0	83.5	**152**	14.9	54.0	18.8	87.7	**252**	42.7	2.5	44.0	89.2
53	62.9	21.0	5.0	88.9	**153**	34.1	25.8	19.0	78.9	**253**	18.9	9.7	44.4	73.0

54	5.6	10.9	5.2	21.7	**154**	45.9	27.6	19.0	92.5	**254**	3.0	21.3	44.7	69.0
55	39.8	35.5	5.2	80.5	**155**	14.3	39.9	19.5	73.7	**255**	0.3	13.1	44.8	58.2
56	1.2	27.7	5.2	34.1	**156**	27.1	17.5	19.7	64.3	**256**	32.3	11.9	45.5	89.7
57	32.8	15.7	5.6	54.1	**157**	50.0	16.8	20.0	86.8	**257**	42.7	6.5	45.8	95.0
58	56.4	1.5	5.7	63.6	**158**	11.6	31.2	20.2	63.0	**258**	26.9	6.4	46.7	80.0
59	6.4	25.1	6.1	37.6	**159**	53.6	13.9	20.3	87.8	**259**	25.2	22.8	47.6	95.6
60	81.9	4.3	6.2	92.4	**160**	6.8	10.4	20.3	37.5	**260**	27.0	13.1	47.9	88.0
61	35.5	22.2	6.3	64.0	**161**	2.4	19.6	20.7	42.7	**261**	11.1	35.1	47.9	94.1
62	66.3	13.0	6.3	85.6	**162**	25.5	8.8	20.8	55.1	**262**	5.0	21.3	48.1	74.4
63	10.9	28.2	6.4	45.5	**163**	15.9	28.3	20.9	65.1	**263**	4.4	6.3	48.3	59.0
64	26.1	8.1	6.6	40.8	**164**	7.5	37.9	21.0	66.4	**264**	4.8	38.2	48.3	91.3
65	7.7	19.0	6.8	33.5	**165**	26.5	30.0	21.0	77.5	**265**	32.6	11.4	48.4	92.4
66	35.0	41.7	6.8	83.5	**166**	18.0	56.5	21.1	95.6	**266**	9.7	34.8	48.5	93.0
67	24.8	18.5	6.9	50.2	**167**	12.6	43.1	21.1	76.8	**267**	9.2	28.8	48.9	86.9
68	5.6	58.6	7.0	71.2	**168**	34.1	25.8	21.2	81.1	**268**	4.1	33.9	49.0	87.0
69	8.6	0.6	7.1	16.3	**169**	14.9	22.8	21.5	59.2	**269**	19.2	20.5	49.4	89.1
70	2.1	18.6	7.1	27.8	**170**	51.7	1.6	21.6	74.9	**270**	5.9	1.4	50	57.3
71	25.3	15	7.2	47.5	**171**	47.8	2.3	21.8	71.9	**271**	40.6	0.2	50.1	90.9
72	51.5	7.5	7.4	66.4	**172**	4.4	26.9	22.2	53.5	**272**	36.2	6.0	50.4	92.6

73	9.4	76	7.5	92.9	**173**	16.3	29.7	22.5	68.5	**273**	1.0	34.9	51.4	87.3
74	16.8	72.1	7.5	96.4	**174**	31.6	34.3	23.0	88.9	**274**	6.7	2.7	52.9	62.3
75	14.5	64.8	7.8	87.1	**175**	0.6	71.9	23.0	95.5	**275**	13.3	3.9	52.9	70.1
76	29.7	42.0	8.0	79.7	**176**	9.3	29.0	23.0	61.3	**276**	4.0	28.5	53.6	86.1
77	3.5	29.5	8.0	41.0	**177**	0.8	41.0	23.1	64.9	**277**	28.3	7.8	55.3	91.4
78	16.1	71.8	8.3	96.2	**178**	20.4	5.7	23.2	49.3	**278**	21.1	8.3	56.2	85.6
79	16.4	55.3	8.7	80.4	**179**	13.8	57.4	23.5	94.7	**279**	11.8	25.5	56.7	94.0
80	28.4	34.6	9.1	72.1	**180**	31.7	10.9	23.5	66.1	**280**	17.5	17	56.8	91.3
81	54.8	9.5	9.1	73.4	**181**	15.0	56.1	23.7	94.8	**281**	31.3	6.7	57.1	95.1
82	20.0	65.5	9.3	94.8	**182**	22.8	34.2	23.9	80.9	**282**	30.2	4.4	58.2	92.8
83	34.2	19.3	9.5	63.0	**183**	30.5	37.9	23.9	92.3	**283**	2.7	24.3	58.9	85.9
84	40.2	34.2	9.7	84.1	**184**	12.1	56.7	24.8	93.6	**284**	27.4	5.2	59.4	92.0
85	11.7	14.8	9.8	36.3	**185**	3.8	22.7	24.9	51.4	**285**	7.1	20.0	60.6	87.7
86	40.9	6.7	10.1	57.7	**186**	16.7	44.4	25.3	86.4	**286**	7.5	6.3	63.0	76.8
87	22.2	25.6	10.3	58.1	**187**	11.4	20.8	25.4	57.6	**287**	3.8	23.7	63.9	91.4
88	29.5	19.8	10.3	59.6	**188**	21.6	17.5	25.6	64.7	**288**	18.1	1.6	65.3	85.0
89	39.8	13.0	10.3	63.1	**189**	26.5	36.4	25.9	88.8	**289**	6.5	10.7	65.3	82.5
90	0.9	42.5	10.7	54.1	**190**	22.4	6.3	26.2	54.9	**290**	2.8	14.7	68.6	86.1
91	6.7	69.1	10.8	86.6	**191**	26.8	20.1	26.4	73.3	**291**	9.7	11.5	69.7	90.9

92	0.6	27.1	11.1	38.8	**192**	16.3	34.8	26.7	77.8	**292**	2.5	20.5	71.0	94.0
93	62.8	18	11.1	91.9	**193**	15.0	10.2	26.9	52.1	**293**	10.4	1.0	74.9	86.3
94	66.3	18.0	11.3	95.6	**194**	33.4	5.9	27.0	66.3	**294**	0.2	16.9	75.1	92.2
95	73.9	7.6	11.4	92.9	**195**	27.2	18.8	27.1	73.1	**295**	9.9	8.2	78.2	96.3
96	21.6	53.2	11.4	86.2	**196**	58.5	2.9	27.3	88.7	**296**	1.3	5.3	79.5	86.1
97	21.3	14.4	11.6	47.3	**197**	11.6	54.9	27.4	93.9	**297**	5.8	9.7	80.2	95.7
98	34.6	0.9	11.6	47.1	**198**	12.3	8.3	27.7	48.3	**298**	1.3	5.2	80.9	87.4
99	51.9	21.6	11.9	85.4	**199**	25.4	35.2	27.8	88.4	**299**	9.4	0.5	81.7	91.6
100	18.3	1.3	11.9	31.5	**200**	14.4	48.0	28.0	90.4	**300**	0.7	11.6	82.0	94.3

Table S2. Sequential model building approach comparing the linear (P, C, L), quadratic (P x P, C x C, L x L) and correlational (P x C, P x L, C x L) effects of nutrient intake on response variables within female (A) and male (B-H) *N. cinerea*. Whenever an overall effect is significant, univariate tests are provided to determine which nutrient(s) contributes to this effect (denoted by letter in superscript, statistics provided in table footer).

	SS_R	SS_C	DF_1	DF_2	F	P
Females						
A. Gestation time vs offspring number						
Linear	485.95	464.67	3	532	8.12	0.0001[A]
Quadratic	419.13	411.77	3	526	3.13	0.025[B]
Correlational	408.82	402.68	3	520	2.64	0.049[C]
Males						
B. 3H2B vs 2MT						
Linear	528.42	525.66	3	574	1.00	0.39
Quadratic	511.75	504.83	3	568	2.60	0.06
Correlational	504.18	494.16	3	562	3.80	0.01[D]
C. 3H2B vs 4E2M						
Linear	520.70	512.47	3	574	3.07	0.03[E]
Quadratic	482.26	476.95	3	568	2.11	0.10
Correlational	470.75	470.27	3	562	0.19	0.90
D. 2MT vs 4E2M						
Linear	536.25	532.94	3	574	1.19	0.31
Quadratic	512.06	505.35	3	568	2.51	0.06
Correlational	504.03	497.69	3	562	2.39	0.07
E. 3H2B vs Attractiveness						
Linear	516.13	516.06	3	583	0.03	0.99
Quadratic	483.79	482.05	3	577	0.70	0.56
Correlational	480.74	473.20	3	571	2.03	0.11
F. 2MT vs Attractiveness						
Linear	538.96	536.52	3	583	0.88	0.45
Quadratic	516.57	510.44	3	577	2.31	0.08
Correlational	501.03	499.62	3	571	0.54	0.66
G. 4E2M vs Attractiveness						
Linear	531.66	523.33	3	583	3.09	0.03[F]
Quadratic	483.85	482.57	3	577	0.51	0.68
Correlational	480.21	475.73	3	571	1.79	0.15
H. Attractiveness vs fertility						
Linear	578.76	547.09	3	592	11.42	0.0001[G]
Quadratic	529.04	517.76	3	586	4.26	0.005[H]
Correlational	504.67	501.87	3	580	5.08	0.002[I]

Univariate tests: [A] P: $F_{1,532} = 22.04$, $P = 0.0001$, L: $F_{1,532} = 4.76$, $P = 0.029$; [B] P: $F_{1,526} = 3.89$, $P = 0.049$; [C] P x L: $F_{1,520} = 4.76$, $P = 0.03$; [D] C x L: $F_{1,562} = 5.24$, $P = 0.022$; [E] P: $F_{1,574} = 5.42$, $P = 0.02$; [F] P: $F_{1,583}=4.71$, $P=0.03$; C: $F_{1,483}=4.11$, $P=0.04$; [G] P: $F_{1,592} = 18.33$, $P = 0.0001$; L: $F_{1,592} = 5.81$, $P = 0.02$; [H] L x L: $F_{1,586} = 10.93$, $P = 0.001$; [I] C x L: $F_{1,580} = 6.02$, $P = 0.014$.

Table S3. Sequential model building approach comparing the linear (P, C, L), quadratic (P x P, C x C, L x L) and correlational (P x C, P x L, C x L) effects of nutrient intake on response variables across the sexes in *N. cinerea*. Whenever an overall effect is significant, univariate tests are provided to determine which nutrient(s) contributes to this effect (denoted by letter in superscript, statistics provided in table footer).

	SS_R	SS_C	DF_1	DF_2	F	P
B. Attractiveness vs offspring production						
Linear	532.96	523.43	3	562	3.41	0.02[A]
Quadratic	497.49	460.42	3	556	14.92	0.0001[B]
Correlational	457.56	452.41	3	550	2.09	0.10
C. Fertility vs offspring production						
Linear	546.72	543.60	3	562	1.08	0.36
Quadratic	525.61	490.51	3	556	13.26	0.0001[C]
Correlational	480.72	475.62	3	550	1.97	0.12

Univariate test: [A] C: $F_{1,562} = 3.56$, $P = 0.04$; [B] P x P: $F_{1,556} = 15.62$; C x C: $F_{1,556} = 24.69$, $P = 0.0001$; [C] P x P: $F_{1,556} = 12.55$, $P = 0.0001$; C x C: $F_{1,556} = 24.85$, $P = 0.0001$.

Table S4. Multivariate Analysis of Variance (MANOVA) examining the effects of diet triplet, sex and their interaction on the difference in intake of protein (P), carbohydrate (C) and lipid (L) intake from that expected in diets in a triplet were consumed at random. Univariate ANOVAs were conducted to determine how each nutrient contributed to the overall multivariate effects.

	MANOVA					**Univariate ANOVAs**			
Source	Pillai's Trace	*F*	*df*	*P*		Nutrient	*F*	*df*	*P*
Diet triplet (A)	2.95	261.14	21,96	0.0001		P	201.42	7,47	0.0001
						C	651.52	7,47	0.0001
						L	459.89	7,47	0.0001
Sex (B)	0.94	157.98	3,30	0.0001		P	5.24	1,47	0.03
						C	341.67	1,47	0.0001
						L	314.59	1,47	0.0001
A x B	2.51	23.23	21,96	0.0001		P	10.97	7,47	0.0001
						C	43.79	7,47	0.0001
						L	34.53	7,47	0.0001
Females									
Diet triplet	2.98	272.01	21,48	0.0001		P	214.73	7,23	0.0001
						C	514.87	7,23	0.0001
						L	624.58	7,23	0.0001
Males									
Diet triplet	2.91	69.73	21,48	0.0001		P	51.22	7,23	0.0001
						C	205.51	7,23	0.0001
						L	101.21	7,23	0.0001

References

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